CRANFIELD UNIVERSITY

SCHOOL OF APPLIED SCIENCES

DOCTOR OF PHILOSOPHY
ACADEMIC YEAR 2011 – 2012
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INTERACTIONS BETWEEN MICROBIAL COMMUNITY STRUCTURE AND PATHOGEN SURVIVAL IN SOIL

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JANUARY 2012

THIS THESIS IS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Abstract

Manure and slurry are valuable resources that may enhance many soil properties. However, organic amendments can pose a significant health risk to both humans and livestock if not managed correctly due to pathogenic loads that may be carried within them. Therefore it is crucial to identify the factors that affect pathogen survival in soil, in order to gain maximum benefit from such resources, whilst minimising the threat to public and animal welfare. This research aimed to elucidate the impact of microbial community structure on pathogen decline following entry of such organisms into the soil. It was hypothesised that pathogen survival would be significantly influenced by both diversity and phenotypic configuration of the microbial community. This was experimentally investigated within three distinctly different biological contexts.

Firstly, it was shown that the survival of *Escherichia coli* O157 was significantly affected by the presence of an intact microbial community. Microcosms consisting of sterile and non-sterile sand and clay soils were inoculated with *E. coli* and destructively sampled over time. The pathogen remained stable at 4^{o}C, irrespective of biological status. However at 18^{o}C, the pathogen grew in sterile soil and declined in non-sterile soil. This result was attributed to microbial antagonism in non-sterile soil, which only became apparent at 18^{o}C, due to increased metabolic activity of the native community.

The next experiment was designed to investigate the impact of microbial diversity and community configuration on the survival of a suite of model pathogens. A gradient of community complexity was created by inoculation of gamma-irradiated soil mesocosms with a serial-dilution of a suspension of a field soil. Soils were incubated to allow biomass equilibration and the establishment of distinct community phenotypes. Sub-samples were then inoculated with *Listeria*, *Salmonella* and *E. coli* strains and survival was monitored over 160 days. Death rates were calculated and plotted as a function of dilution, which represented diversity, and of principal component (PC) scores from PLFA profiles, which represented the phenotypic community context. There was some evidence of a diversity effect as weak negative linear correlations were observed between death rate and dilution for *S.* Dublin and environmentally-persistent *E. coli*. However, a much stronger correlation was observed between death rate and certain PC scores for these organisms. No effect of diversity or phenotype was detected on either *L. monocytogenes* or *E. coli* O157. These results suggest that pathogen survival was affected by diversity, but the phenotypic community context was apparently much more influential. Additionally, such community effects were specific to pathogen type.

Pathogen survival was also investigated in the context of highly-contrasting communities within a range of naturally-derived field soils. PLFA analysis was used to determine phenotypic community structure and soils were also characterized for a range of physico-chemical properties. They were inoculated with *Listeria*, *Salmonella* and *E. coli* strains as above. Pathogen survival was monitored over 110 days and death rates were calculated. Physicochemical and biotic data, including PC scores derived from PLFA profiles, were used in stepwise regression analysis to determine the predominant factor influencing pathogen-specific death rates. PC scores were identified as the most significant factor in pathogen decay for all organisms tested, with the exception of an environmentally-persistent *E. coli* isolate.

Overall, these results demonstrate the importance of soil biological quality, specifically the configuration of the microbial community, in pathogen suppression, and provide a possible means to assess the inherent potential of soils to regulate pathogen survival. This may lead to the identification of management strategies which will ultimately accelerate pathogen decay, and therefore improve the safety of agricultural practice.

Acknowledgements

First and foremost, I would like to thank my super supervisors Karl Ritz, Sean Tyrrel, Karl Richards and Fiona Brennan for their wonderful support both scientifically and at times emotionally. I laughed, I cried, I moaned (a lot!) but I never felt alone and for that I am truly grateful. I would also like to thank Bryan Griffiths for his patience and understanding in the preparation of this document. Thanks to the technical staff at both Cranfield University and Teagasc, who were always helpful and pointed me in the right direction. I would also like to gratefully acknowledge the Teagasc for funding this project under the Walsh Fellowship scheme.

This experience would not have been the same without the amazing friends that I've made along the way, who are too many to mention. In particular, thanks to my 'Cranfield Cluster' buddies, Niamh Rogers, Paul Massey and Fred Bourdin, and office-mates Diana Selbie and Enda Cahalan. You were a constant source of support and entertainment! Thanks also to my non-PhD friends for listening to my endless ramblings and keeping me sane.

Thanks to my Nanny Roche for countless cups of tea, and to Sarah, for being such a wonderful sister, and for talking me off the ledge on more than one occasion!

To my parents, Aidan and Elaine, you always made me feel like I could achieve anything and encouraged me to reach for the stars. To my Mum in particular, you were there for me day and night these last three years, and I honestly could not have come to this point without your unconditional love and support. Thank you.

To Tom, you were my rock throughout this entire process, even though I fully admit I was a nightmare most of the time…..little did you know what you were signing up for! Thanks for sticking by me and for being my best friend.

Finally, I would like to dedicate this thesis to the memory of my Uncle Paul. You are gone but by no means forgotten. Thank you for being an inspiration.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	Analysis of variance
CEC	Cation exchange capacity
CFE	Chloroform fumigation extraction
CFU	Colony forming unit
CLPP	Community level physiological profiling
CT-SMAC	Cefixime Tellurite-Sorbitol MacConkey agar
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribose nucleic acid
EAEC	Enteroaggregative E. coli
EHEC	Eneterohemorrhagic E. coli
EIEC	Enteroinvasive E. coli
EPEC	Enteropathogenic E. coli
ERIC	Enterobacterial repetitive intergenic consensus
ETEC	Eneterotoxigenic E. coli
GC	Gas chromatograhpy
HEPA	High efficiency particulate air
HUS	Hemolytic uremic syndrome
IC	Inorganic carbon
LB	Luria-Bertani
MAC	MacConkey
OM	Organic matter
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic acid
SIR	Substrate induced respiration
SMAC	Sorbitol MacConkey
SMBC	Soil microbial biomass carbon
STEC	Shiga toxin-producing E. coli

TC	Total carbon
TOC	Total organic carbon
T-RFLP	Terminal restriction fragment length polymorphism
UV	Ultraviolet
VBNC	Viable but not culturable
VTEC	Vero toxin-producing E. coli
WHC	Water holding capacity
XLD	Xylose Lysine Deoxycholate

Chapter 1. Introduction and literature review

1.1. Introduction

Following agricultural intensification post-World War II, the use of mineral fertilisers became popular due to an associated increase in yield. This led to a reduction in land application of organic 'waste' materials such as livestock manures. There was also a tendency towards specialised rather than mixed farming systems, and so manure became less available. Typically these agronomic systems placed an emphasis on production, and little consideration was given to environmental sustainability. Recently, however, there has been a fundamental shift in societal thinking regarding agricultural practice, which has seen a reversion to land disposal of so-called 'organic waste' and renewed appreciation of the value of this resource (Østergård et al., 2009). Manure and slurry contain nutrients and minerals intrinsically essential for plant growth that, if correctly applied, can increase soil fertility, soil condition and enhance crop yield (Fenlon et al., 2000). Additionally they can improve soil tilth and reduce erosion rates by up to 70% (Ramos et al., 2006). However, problems may arise when manure is applied excessively whilst crop requirements for nutrients are low. This can cause leaching of soluble nutrients beyond the plant/root zone to both surface and ground waters, often resulting in eutrophication and degradation of water quality (Smith et al., 2001a; Smith et al., 2001b). Also, faecal waste typically contains numerous pathogenic organisms, including bacteria, viruses and protozoa, which can be harmful or even fatal if ingested by humans (Mawdsley et al., 1995). Ingestion of these enteric pathogens by livestock can also cause re-infection of animals and result in economic loss to agriculture. Therefore, it is desirable to maximise the fertiliser potential of manure whilst minimising contamination of the wider environment. An important aspect of such minimisation is the identification of both biotic and abiotic factors that contribute to pathogen decline, which may allow for more effective management of the pathogen load and consequently reduced pathogen losses from soil to crops and water. This would also reduce transmission potential among humans and livestock, resulting in improved public health and agricultural practice.

The aim of this chapter is to summarise key work on pathogen survival in soil and identify areas in this field which necessitate further research. It provides an overview of the microbial composition of livestock wastes and describes various pathogenic microorganisms of clinical significance. Pathogenic exposure routes and the cycle of animal and human re-infection by pathogens derived from the agricultural environment are discussed in detail. Current opinion on physical, chemical and in particular, biological factors which influence pathogen survival is reviewed, and methods of manure management for the purposes of pathogen removal are also discussed. Finally, the practice of intentional release of beneficial microorganisms is described in relation to effective management of pathogen loads.

1.2. Composition of livestock manure

Public perception of organic materials generated by livestock has been historically variable. Prior to the industrial revolution, these materials were viewed as a resource, and appreciated for their inherent nutrient value. However, industrialisation led to an overt reliance on mineral fertilisers, and livestock manure became negatively viewed as problematic waste outputs. With demand for global crop production estimated to increase by 100-110% between 2005 and 2050 (Tilman et al., 2011), it is crucial that there is an emphasis on *sustainable* agricultural intensification. Thus, there is a need to change public perception of these materials previously regarded as waste, which could contribute toward achieving this sustainability.

Organic fertiliser that is generated on the farm is defined by the EC (Good Agricultural Practice for Protection of Waters) Regulations, 2010 (S.I. 610/2010) as '*any fertiliser other than that manufactured by an industrial process and includes livestock manures, dungstead manure, farmyard manure, slurry, soiled water and silage effluent*' (EC, 2010). The quality of this resource is significantly influenced by feeding regimen, species and age demographics of the herd, the method of collection and the volume of water added (Zhang and Westerman, 1997). In addition, the bedding material and storage conditions can also determine the composition of the final resource output.

Livestock manures, which are defined as '*waste products excreted by livestock or a mixture of litter and waste products excreted by livestock, even in processed form*'

(EC, 2010) contains excreta plus numerous secretions from the nose, throat, vagina, mammary glands, skin and placenta (Pell, 1997). Livestock manure can be subdivided into categories based on dry matter content and method of collection used (Burton and Turner, 2002).

The EC (Good Agricultural Practice for Protection of Waters) Regulations, 2010 (S.I. 610/2010) also give a comprehensive description of different livestock manure types. Soiled water, which is the most dilute form, includes '*waters from concreted areas, hard standing areas, holding areas for livestock and other farmyard areas where such water is contaminated with:*

- *Livestock faeces or urine or silage effluent*
- *Chemical fertilisers*
- *Washings such as vegetable washings, milking parlour washings or washings from mushroom houses*
- *Water used in washing farm equipment*' (EC, 2010).

In some cases, concentrated effluents from slurry and manure stores are treated as dirty water, giving rise to variable quality and composition based on individual farm practice (Brewer et al., 1999). Slurry, which has a higher dry matter content as compared to soiled water, is defined as '*any excreta produced by livestock while in a building or yard, and a mixture of such excreta with rainwater, washings or other extraneous material, or any combination of these, of a consistency that allows it to be pumped or discharged by gravity at any stage in the handling process, but does not include soiled water*' (EC, 2010). Finally, the most solid form of livestock manure is classed as farmyard manure, which is defined as '*a mixture of bedding material and animal excreta in solid form arising from the housing of cattle, sheep and other livestock, excluding poultry*' (EC, 2010). Livestock manures are stored and treated prior to land disposal, and this will be discussed in detail in Section 1.8.

1.3. Pathogens associated with livestock manures

Livestock manures contain a diverse pathogenic load. The main groups of interest include bacteria, e.g. *E. coli* and *Salmonella*, protozoa, e.g. *Cryptosporidium* and *Giardia* and viruses, e.g. Enterovirus and Rotavirus (Bicudo and Goyal, 2003). Manures can also contain parasitic intestinal worms called helminths e.g. *Ascaris* and *Trichuris*; however helminth infection is mostly confined to tropical and subtropical regions (Bethony et al., 2006). Hutchison et al., (2004a) carried out a survey to investigate the concentration of common zoonotic pathogens in livestock manure produced in the UK. The highest level of $2x10^8$ colony forming units (CFU) g^{-1} was recorded for *E. coli* O157 in cattle manure. Overall, recovery was variable and depended on manure and pathogen type. Physiology, survival and infection characteristics of the main pathogen groups associated with livestock manure are summarised below.

1.3.1. Bacteria

Escherichia coli, *Campylobacter* spp., *Salmonella* spp. and *Yersinia* spp. are the main pathogenic bacteria shed by grazing livestock. Despite the fact that *E. coli* is a natural component of both human and animal intestinal microflora, a number of enterovirulent strains do exist, namely enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), vero cytotoxigenic (VTEC/STEC), enteroaggregative (EAEC) and diffusely adherent (EAEC) (EFSA, 2007). VTEC produce cytotoxins that cause inhibition of cellular protein synthesis within eukaryotic organisms. Enterohemorrhagic *E. coli* (EHEC) describes any VTEC that are highly pathogenic toward humans (EFSA, 2007). As few as 100 EHEC organisms can cause infection (WHO, 2006). VTEC serotype O157 is particularly toxic and can induce bloody diarrhoea and haemolytic uraemic syndrome (HUS) in humans, which can result in renal failure and haemolytic anaemia (Porter et al., 1997). Human infection is predominantly caused by this serotype; however infection by serotypes other than O157, namely O26, 111, 103 and O145 is being reported with increasing frequency. A large outbreak of HUS occurred in Germany in May 2011 and this was associated with a Shiga-toxin producing O104 strain (Böhmer et al., 2011). These serotypes are grouped together and classed as VTEC non-O157 (EFSA, 2007). Cattle have been identified as the primary source of VTEC

among livestock (Chapman et al., 1997; Jones, 1999); however it has also been isolated among sheep, goats, pigs and chickens (WHO, 2006). *E. coli* pathogenicity is host-specific. This explains why commensal *E. coli* can colonise and reside in the human intestine from birth, whereas other strains, such as O157, are capable of colonising livestock without causing illness but can have severe health implications for humans following ingestion (Jones, 1999). Shedding of VTEC O157 is variable and Wang et al., (1996) found that the shedding rate ranged between 10^5 and detection by enrichment only in a survey of dairy calves. Human infection by VTEC O157 is primarily caused by consumption of contaminated food, water and increasingly, crops fertilised with untreated manure (Duffy, 2003; FSAI, 1999). Raw milk from a healthy animal typically has fewer than microbial cells per millilitre (Chye et al., 2004); however a lack of basic sanitation in the milking, storage and transportation processes can significantly increase this load (Aumaitre, 1999).

Campylobacter spp. have been identified in the intestines of pigs, cattle, sheep and poultry, and are one of the most common causes of food-associated gastroenteritis (Cools et al., 2003). This Gram-negative bacterium requires reduced O_2 and increased CO_2 relative to atmospheric conditions for survival and is highly infectious, with as few as 1000 cells required to induce illness in humans (WHO, 2006). Infection and symptomatic bacterial gastroenteritis, termed 'campylobacteriosis', is commonly attributed to *C. jejuni* species, contracted by the ingestion of contaminated meat and dairy products and also by the ingestion of contaminated water.

Salmonella are Gram-negative rod-shaped members of the Enterobacteriaceae family (Mawdsley et al., 1995). This bacterium has a wide host range in the environment and has been isolated from humans, poultry, cows, pigs, birds and reptiles. Some strains show host specificity e.g. *S. typhi*, which is confined to humans, however most show high potential for transmission, which typically occurs via the faecal-oral route. The dominant mode of transmission is via the consumption of contaminated food, and also, to a lesser extent, the ingestion of contaminated water. Human infection is primarily attributed to *S. enteritidis* spp., which can induce diarrhoea and systemic infections (Rosen, 2000).

Both animals and humans are implicated in the transmission of *Yersinia*, which is a member of Enterobacteriaceae family. Human infection is caused by ingestion of

pathogenic *Y. pestis*, *Y pseudotuberculosis* and particularly *Y. enterocolitica* spp., which is commonly detected in pigs. Again, this pathogen is spread via the faecal-oral route, and is contracted via the ingestion of contaminated food and water (WHO, 2006).

1.3.2. Protozoa

Pathogenic protozoa of primary importance in manure include *Cryptosporidium spp.* and *Giardia spp.*, both of which are transmitted via the faecal-oral route among animals and humans. The lifecycles of both are similar – when excreted to the environment, they form oocysts and cysts respectively, which can remain viable for months in cool moist conditions. Following ingestion by a suitable host, the oocyst, which represents the infectious stage, reacts with gastric acid in the gut and excystation occurs. This results in the release of *Cryptosporidium* sporozoites and *Giardia* trophozoites, and illness ensues (Thompson et al., 2008). *Cryptosporidium* is an obligate parasite, and therefore cannot multiply outside the host, but can survive and remain infective in soil for greater than 12 weeks depending on prevailing environmental conditions (Olson et al., 1999). Other work by Jenkins et al., (2002) investigated oocyst longevity in soil, in relation to soil type, temperature and water potential. Findings showed that oocysts could remain viable in the soil from several months to a few years under the experimental conditions tested. The main source of human infection is *C. parvum*, which can cause vomiting and fever; however the disease is self-limiting and typically becomes contained within a week of infection in healthy individuals. Nonetheless, cryptosporidiosis has the potential to become life-threatening in the case of immuno-compromised people (Mac Kenzie et al., 1994; Atwill, 1996). Humans and livestock, particularly young animals, are a major source of *Cryptosporidium* in the environment (WHO, 2006).

The *Giardia* spp. typically associated with human illness is *Giardia lamblia*. This flagellate trophozoite multiplies in the gastrointestinal tract, and forms a cyst which is excreted intermittently to the environment. Limited research has been carried out on *Giardia* survival in soil (Robertson and Gjerde, 2004). However, it has been shown that *Giardia* is more susceptible to environmental extremes, and is therefore less persistent than *Cryptosporidium* (Keeley and Faulkner, 2008). A study carried out by Olson et al., (1999) showed that *Giardia* cysts could remain infective in soil environment for up to 7

weeks. Human infection may be asymptomatic, however in many cases, *G. lamblia* will cause vomiting and diarrhoea in the infected individual. Again the disease is self-limiting and should rectify in a short space of time among healthy individuals (WHO, 2006). *G. lamblia* are prevalent in livestock manure, and it was found that concentrations of this organism ranged from 10^3 to 10^6 per litre of runoff from different manure types. The highest numbers were noted for fresh cattle manure and the lowest for aged cattle manure, with swine manure slurry generating intermediate runoff concentrations (Thurston-Enriquez et al., 2005).

1.3.3. Viruses

Various viruses are also shed in livestock excreta, and of all pathogenic microorganisms, they display the greatest resistance to environmental conditions. Their presence in the environment is difficult to predict using conventional methods based on faecal coliforms, which are geared specifically toward bacterial identification (Malik, 2004). Gastrointestinal illness in humans is generally caused by the ingestion of enteropathogenic viruses, such as astroviruses, caliciviruses and rotaviruses. Non-enteropathgenic viruses, e.g. Hepatitis A and E, adenoviruses and enteroviruses, are also found to reside in the intestinal tract of the host; however this type of virus does not manifest as gastroenteritis (Guardabassi et al., 2003). Once again, contaminated food is the dominant causative agent of illness, with little evidence to support that water is a significant medium for transmission to humans (Rosen, 2000). Viruses are obligate parasites that cannot effectively multiply outside the host, and transmission is limited by a narrow host range (Pell, 1997). The risk of human illness is mostly associated with the application of biosolids and human wastewater to agricultural land, and enteric viruses released to the soil environment in this manner include Hepatitis A, Hepatitis E, Poliovirus Types 1 and 2, adenoviruses, echoviruses and coxsackievirus (Santamaría and Toranzos, 2003). Rotavirus can cause mild to severe diarrhoea in humans and in some cases can result in death due to fatal dehydration (Mawdsley et al., 1995). Rotavirus is also the leading cause of life-threatening diarrhoeal disease in infants (Parashar et al., 2006). Hepatitis E Genotype IV has been isolated from both humans and pigs in China, and therefore pig farming and disposal of associated manures may

potentially constitute a reservoir for this virus and play a role in human transmission. The virus is contracted via contact with pigs or pig manure and those directly involved in pig farming or living downstream from such farms were found to be 74% and 29% more likely to acquire infection respectively. Although this strain is asymptomatic, there is now concern regarding the phylogenetic similarity between Hepatitis E Genotype III among human and pig isolates. This genotype is more widely distributed than Genotype IV which is typically found in South East Asia, and therefore development of Genotype III virulence could have important consequences for pig farming worldwide (Zheng et al., 2006).

1.4. Pathogen exposure

A conceptual model of pathogen transmission within the agricultural environment is presented (Fig. 1.1). Microbial pathogens are released in faecal waste of both animals and humans, and enter the soil environment either **directly** via faecal shedding, or **indirectly** via the application of slurry and sewage sludge. In addition to livestock, wild animals and birds contribute to pathogen load in the environment (Jones, 2001; Jiang et al., 2007; Benskin et al., 2009), and there is also evidence to suggest that pathogens can exist as naturalised populations within the soil matrix (Texier et al., 2008; Brennan et al., 2010a).

The rate of shedding is an important determinant of direct pathogen loss to soil. Pathogen shedding is governed by various factors such as animal stress, herd health, farm hygiene, antibiotic use, age demographic, pathogen exposure, feeding regime, and biological and physico-chemical conditions within the gut (Russell and Rychlik, 2001; Rugbjerg et al., 2003; Chase-Topping et al., 2007). Indirect pathogen loading via application of livestock manure is influenced by the initial concentration of pathogens shed, and management factors such as storage (Tyrrel and Quinton, 2003), treatment (Bicudo and Goyal, 2003), and method and timing of manure applications (Avery et al., 2004; Holley et al., 2006).

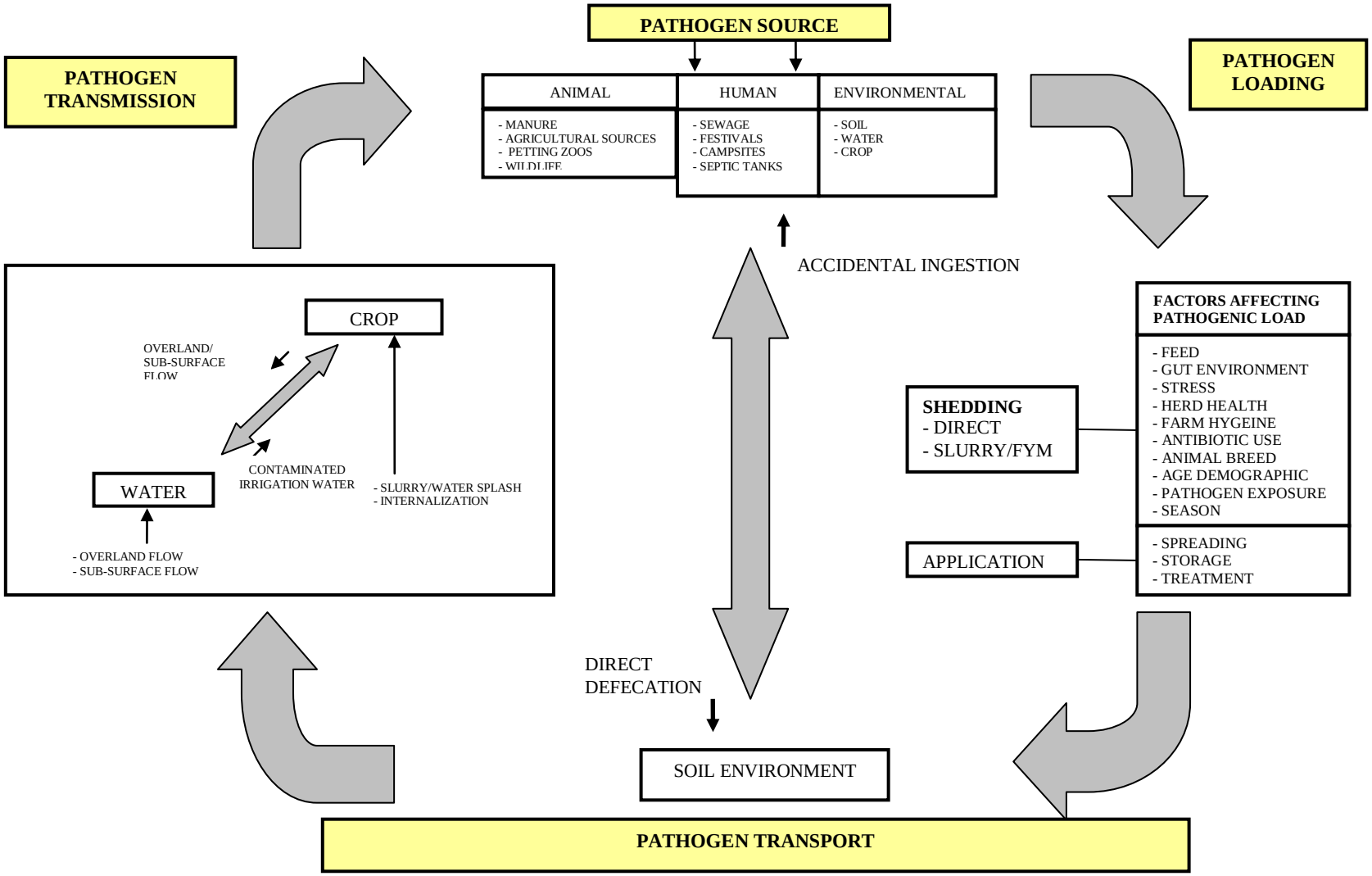


Fig. 1.1 Conceptual model of animal/ human re-infection with pathogens derived from the agricultural environment

1.4.1. Legislation

Organic amendments, including livestock manure and sewage sludge, are treated according to legislative guidance primarily devised to achieve efficient and sustainable nutrient use. This also has implications for pathogen loads in the final organic output, and potential for transmission following land application.

At a European level, the use of livestock manure in agriculture is regulated by the Council Directive 91/676/EEC (EC, 1991), concerning the protection of waters against pollution caused by nitrates from agricultural sources. This is transposed into Irish law under the European Communities (Good Agricultural Practice) for Protection of Waters Regulations S.I. 610/2010 (EC, 2010). These Regulations specify storage requirements and spreading restrictions to prevent diffuse and point source contamination of adjacent surface and groundwaters. According to these Regulations, there should be adequate storage for 18-26 weeks worth of manure produced on the farm, depending on the type of livestock and farm location. Additionally, spreading is prohibited during the winter months when rainfall is high, and is not permitted when the land is steeply sloping, waterlogged, flooded, frozen, and snow-covered or in the event of an adverse weather-forecast within 48 hours, to prevent loss of nutrients and similarly, pathogens to water. Spreading must not be carried out within a specified distance of a watercourse, and this perimeter is determined by the type of watercourse and the nature of its use.

The safe use of sewage sludge for agricultural purposes is regulated by the Council Directive 86/278/EEC (EC, 1986), concerning the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Again, this has been adopted by the Irish government according to the Waste Management (Use of Sewage Sludge in Agriculture) Regulations S.I. 261/2001 (EC, 2001). These regulations focus on crop nutrient requirements and preserving the quality of soil, surface water and groundwaters. In addition, a soil sampling strategy is outlined for testing parameters such as dry matter, organic matter (OM), pH, nitrogen (N) and phosphorus on land where sludge has been applied. Maximum permissible concentrations of various heavy metals in both soil and sludge are also indicated. In addition, there are stringent restrictions on spreading sludge on land used for crop production and grazing.

Although this legislation outlines general provisions for environmental protection, there is no specific requirement to achieve pathogen reductions in either livestock manures or sewage sludge prior to land disposal, nor is it required to monitor for pathogens in soil following amendment.

In the UK, guidance regarding sludge treatment for pathogen reduction is better defined. The Safe Sludge Matrix (2001) outlines the restrictions on sewage sludge use in agriculture. All sludge, with the exception of sludge intended for use on non-food crops, must be conventionally treated by means of biological, chemical or heat treatment prior to use in order to achieve a 99% reduction in pathogens. Sludge treated to this standard must be deep-injected and spreading on the surface is not permitted. Harvesting of fruit or salad crops that might be eaten raw must not take place for 30 months following the application of conventionally-treated sludge. This restriction is less stringent for vegetable crops which are subject to a 12 month lag-time between application and harvest. However, when the sludge has received enhanced treatment to ensure the destruction of 99.9% of pathogens, these crops can be harvested within 10 months of application. In the case of both conventional and enhanced treated sludge, there must be a 3-week interval between sludge application and grazing or harvesting of forage crops to prevent re-infection of livestock (ADAS, 2001).

A number of recommendations to ensure effective minimisation of pathogens in livestock manure have been devised by Nicholson et al., (2004), based on reported pathogen survival times in soil; however there is no legislative requirement to implement these recommendations. Slurry and solid manure should be stored for one and three months respectively, prior to application. If possible, manure generated by young animals that are considered at high risk of pathogen shedding should be dealt with separately. Slurry spreading should be carried out in a way that minimises the risk of aerosol production, which could potentially lead to infection of farm workers by inhalation and serve as a diffuse source of contamination in the wider environment. Crops which have been fertilized by manure should not be harvested within 6 months of application, and similarly, this lag-time should be applied to harvesting of crops on land previously grazed by livestock. Additionally to minimise pathogen transfer between livestock, infected animals should be separated from healthy livestock, and manure application to pasture during the grazing season should be avoided. If this is not

possible, manure must be subjected to storage for one month, and grazing should not be carried out on spread pasture for at least one month post-application (Nicholson et al., 2004). The guidance and regulations have been developed based on the best available information regarding pathogen inactivation. However, most of the studies that have been carried out in this area have focused on abiotic interactions in the soil, not taking into account the inherent diversity and structure of the soil community. Therefore, there is a need to identify the full extent of these biotic interactions to ensure that the legislation is relevant and accurate, and prevent an over-estimation of the threat posed by pathogens, particularly in biologically-diverse soils.

1.4.2. Livestock manure application

Pathogens can be introduced indirectly to the soil by land application of livestock manure. The method of manure application can also affect the likelihood of viable pathogen transmission. Organic fertiliser can be injected into the soil, or surface applied, and there are pros and cons associated with each method. When left on the surface, pathogens are exposed to the full force of fluctuating environmental conditions, which has been shown to increase the inactivation rate as compared to immediate incorporation into the soil (Hutchison et al., 2004a; Nicholson et al., 2004). Additionally, Avery et al., (2004) found that *E. coli* O157 survived on surface vegetation for 6 weeks, but persisted for up 8 weeks when injected into the sub-surface. Band-spreading and injection of slurry is currently gaining popularity due to the associated reductions in greenhouse gas emissions as compared to surface spreading (Nicholson et al., 2004; Rodhe et al., 2006). However, there is evidence to suggest that sub-surface application can result in greater pathogen loss to groundwater (Mawdsley et al., 1995; Semenov et al., 2009; Forslund et al., 2011). This method may also buffer pathogens from environmental stressors such as UV and temperature fluctuations, and thus a potential trade-off exists between environmental and public health concerns and risks.

1.4.3. Transmission

Following pathogen release to the environment, there are three scenarios which can lead to livestock and human infection, which are contingent on the pathogen's ability to

survive and maintain viability outside of the host. Firstly, pathogens may be transmitted by direct contact with contaminated surfaces and accidental ingestion of faeces or contaminated soil particles. This type of infection can occur on the farm (Davis et al., 2005), or during recreational visits to petting zoos (Heuvelink et al., 2002a), campsites (Ogden et al., 2002) and music festivals (Crampin et al., 1999). Williams et al., (2005) investigated the persistence of *E. coli* O157 on various farm surfaces and found that the rate of decline was dependent on the surface material. It was also shown that 6.4% of a contaminated faecal sample that was spread on a gate surface was transferred to a human hand on contact, thus demonstrating the potential of the hand in pathogen transmission (Williams et al., 2005).

Secondly, pathogens may be transported via overland or sub-surface flow to surface and ground waters, and infection may arise via ingestion of contaminated water or accidental ingestion of contaminated recreational water. A famous example is provided by the so-called Milwuakee Outbreak in 1993, during which over 400,000 people became infected by *Cryptosporidium parvum*. Oocyst-contaminated water withdrawn from Lake Michigan did not receive adequate treatment, leading to the largest outbreak in the history of the US (Hoxie et al., 1997). Another waterborne outbreak associated with the public water supply occurred in Walkerton, Canada in 2000, resulting in 2300 reported gastrointestinal cases and 7 fatalities. This outbreak was associated with raw water contamination by *E. coli* and *Campylobacter* leached from land-spread cattle manure during heavy spring rainfall, in conjunction with inadequate water treatment (Hrudey et al., 2003). A survey of potable water supplies in the United States showed that 55% and 16% of samples contained *Cryptosporidium* and *Giardia*, respectively (Rose et al., 1991). This work also showed that contamination was mostly associated with water receiving sewage and agricultural discharge as opposed to 'pristine' water. Pigs are also associated with carriage of *Cryptosporidium* (Ramirez et al., 2004); however, in Ireland, pigs are housed throughout the year (Hyde et al., 2003), and thus the real threat of infection associated with pig slurry exists solely after land-spreading events. The identification of *Cryptosporidium* spp. in 25 of 56 slurry samples taken from 33 Irish pig farms demonstrates the potential for oocyst loss to surface and groundwater following land-spreading under adverse weather conditions (Xiao et al., 2006). In March 2007, Cryptosporidium was implicated in a large Irish outbreak, when

oocyst-contaminated water drawn from Lough Corrib, Galway, was used for public supply, causing infection in approximately 200 Irish people. The majority of cases confirmed were attributed to *C. hominis*, suggesting that contamination originated from a human source in this instance (Pelly et al., 2007). Smith et al., (2006) carried out a comprehensive review of reported outbreaks of waterborne infectious disease in England and Wales between 1993 and 2002. These were mostly associated with *Cryptosporidium* spp. followed by *Campylobacter* spp., and were transmitted through swimming pools and both public and private water supplies.

Finally, it is possible that viable pathogens could be present on the crop surface following waste application, or may become internalised within the crop tissue where they are protected from conventional sanitization (Itoh et al., 1998; Solomon et al., 2002). In this case, a person may become infected if they consume the contaminated produce. The greatest pathogen risk is associated with fresh produce that is eaten raw, e.g. ready-to-eat fruit and vegetables, in particular leafy vegetables and bean sprouts. Leifert et al., (2008) highlighted a number of risk reduction points to improve the microbiological quality of ready-to-eat crops, which focus on six key areas including animal husbandry, outdoor livestock management, manure storage and processing, soil management practices, timing of manure application and irrigation. Despite these recommendations, Leifert et al., (2008) acknowledged that good farm practice alone cannot eliminate the microbiological risk associated with ready-to-eat crops, and that good hygiene in food preparation at home is crucial to prevent infection. An outbreak of shiga-toxin producing *E. coli* serotype O104 occurred in Germany in May 2011, resulting in increased incidence of HUS cases. Initially the outbreak was associated with raw tomatoes, cucumbers and leafy salads (Frank et al., 2011); however it was later confirmed that bean sprouts were the most likely source of *E. coli* infection (Böhmer et al., 2011). A study carried out by Mukherjee et al., (2004) investigated the pathogen load carried by fresh produce associated with different farming practices. A number of samples were collected from organic and conventionally farmed produce in Minnesota, and were tested for the prevalence of *E. coli*. It was found that *E. coli* was significantly more prevalent in uncertified organic produce samples (9.7%) as compared to conventional produce samples (1.6%). However, the prevalence in samples from certified organic farms (4.3%) did not differ significantly from the conventional result.

The maturity of the organic materials used may have accounted for this observation, as fertilisation with fresh animal manure resulted in 19 times the *E. coli* load found on produce fertilised with stored and aged manure (Mukherjee et al., 2004). Franz et al., (2008a) conducted a study to investigate the probability of lettuce contamination with *E. coli* O157 caused by growth in manure-amended soil in the Netherlands. They concluded that typical agricultural systems produced 0.34 contaminated lettuce heads per hectare, amounting to 199 contaminated lettuce heads annually. This increased to 521 for intensive manure use, and decreased to 59 for extensive organic systems. Based on these data, the authors recommended a minimum manure storage time of one month and a fertilisation-to-planting interval of 30 days (Franz et al., 2008a).

1.5. Pathogen shedding

The pathogen load shed by livestock is variable and intermittent, and can be influenced by many factors relating to animal husbandry, and the physical condition of the animal itself (Russell and Rychlik, 2001; Chase-Topping et al., 2007).

Jones et al., (1999) found that the number of *Campylobacter* shed by sheep was highest during lambing, weaning and transport to new pasture, and lowest during gestation. The same study found that stress associated with shearing or dipping did not have any effect; however, stress caused by handling has been noted as a possible cause of shedding among pigs (Dowd et al., 2007). The age of the animal may also be related to the number of microorganisms shed to the environment. For example, Rugbjerg et al., (2003) found that shedding of *E. coli* O157 was more prevalent among calves aged 3 to 6 months as compared to older cattle. Similarly, a study by Nielsen et al., (2002) found shedding of *E. coli* O157 to be highest among calves aged 2 to 6 months, with lower incidences found in calves less than two months old and adult cattle.

Livestock diet plays an important role in rumen microbial ecology, and consequently, much work has been carried out to investigate the effect of different feed types on pathogen shedding. Gilbert et al., (2005) showed that a diet comprised primarily of roughage and molasses reduced shedding of *E. coli* in cattle. This feeding regimen also reduced virulence factors of the EHEC strain, thus reducing pathogenicity both inside the gut and when shed to the environment. Conversely, cattle finished on

grain were more likely to shed *E. coli*. This was because grain decreased pH within the gut of the animal, which favoured the proliferation of acid-tolerant *E. coli* strains (Gilbert et al., 2005). Diez-Gonzales et al., (1998) also found significantly greater concentrations of *E. coli* O157 in the colonic digesta of cattle that were maintained on grain as compared to hay, i.e. 10^6 cells and less than 20,000 cells g^{-1} of digesta, respectively. Additionally, by comparing colonic fatty acid accumulation and pH, they demonstrated that the grain-based diet supported acid-resistant populations of *E. coli* O157. In contrast, hay, which created basic conditions in the gut, supported cells that were particularly susceptible to acid-shock; consequently, survival in the highly acidic human stomach following ingestion would likely be compromised. Therefore, Diez-Gonzales et al., (1998) suggested briefly supplementing cattle feed with hay prior to slaughter to reduce the risk of human infection with *E. coli* O157.

However other work has shown that hay, comprised of either alfalfa or grass, can prolong the duration of shedding. *E. coli* O157 was isolated from cattle faeces for 4, 34 and 42 days following a diet of grain, alfalfa and grass, respectively. It was also found that both the concentration and acid resistance of *E. coli* O157 cells shed by cattle were unaffected by diet (Hovde et al., 1999). A study carried out by Kudva et al., (1997) investigated the effect of diet, change in diet and fasting on shedding of *E. coli* O157:H7 by experimentally inoculated sheep. It was found that a diet of grass resulted in the excretion of twice as many *E. coli* O157:H7 as compared to a corn/alfalfa mix. Grass also resulted in a longer shedding period. In addition, shedding increased following a shift from corn/alfalfa to grass, but a decrease was observed for the opposite dietary sequence. Withholding food and water for a 24 hour period did not induce a greater shedding rate among the animals tested (Kudva et al., 1997).

In light of the conflicting shedding rates associated with different feed types, care must be taken when manipulating feeding regiments with the intention of minimizing pathogen risk.

Aside from diet composition, the physical properties of the feed used may also impact on the physico-chemical environment, and consequently, the microbial ecology of the gut. A study by Mikkelsen et al., (2004) demonstrated that pigs fed on a coarsely ground meal in non-pelleted form promoted specific conditions, namely high dry matter content

and increased pH, that favoured the death of *Salmonella* Typhimurium in the gastrointestinal tract of the animal. Furthermore, diet will also influence manure composition which will partly determine the fate of pathogens shed to the environment. For example, Franz et al., (2005) showed that decline in *E. coli* O157:H7 was higher in cattle manure derived from a pure straw diet than in a diet comprised of high digestible grass silage and maize silage. Although *S.* Typhimurium was more persistent, a similar trend in decline was observed. This was attributed to the high pH and fibre content of the manure produced following the straw feeding regimen, which was not conducive to pathogen survival.

By understanding the factors that govern pathogen shedding, appropriate management and animal husbandry practices can be employed to reduce the release of zoonotic agents to the environment.

1.6. Pathogen survival

The risk associated with enteric pathogens shed by livestock is largely determined by their ability to survive outside the host animal in the soil environment. Survival is determined by both abiotic and biotic parameters. The major physico-chemical factors that influence the survival of introduced microorganisms in soil are currently considered to be soil texture and structure, pH, moisture, temperature, UV irradiation, nutrient and oxygen availability, and land management regimes (reviewed in van Elsas et al., (2011)). Pertinent biotic interactions include antagonism from indigenous microorganisms, competition for resources, predation and occupation of niche space (van Elsas et al., 2002). Pathogen-specific biotic factors that influence survival include physiological status and initial inoculum concentration (van Veen et al., 1997).

1.6.1. Abiotic considerations

1.6.1.1. Soil texture and pH

Soil texture is largely dependent on the nature of the parent material and rate of weathering processes. It is defined by relative proportions of clay, silt and sand.

The ratios of these materials influence the inherent cation exchange capacity (CEC) of the soil, and also influence some aspects of the nature and dynamics of soil structure (Bronick and Lal, 2005). Cation exchange capacity is also largely influenced by clay mineralogy and organic matter. A study by Drake and Motto (1982) showed that variation in CEC between different soil types was primarily associated with clay and organic matter content. Fine-textured clay soils tend to be characterised by a well-developed microstructure with high surface area and high net negative charge. This is associated with availability of habitat, water and nutrients for resident soil microflora (England et al., 1993). Texture also greatly affects soil hydrology and can therefore influence the development of preferential flow paths that aid the transport of pathogens through the soil matrix (Jamieson et al., 2002). Additionally, nutrient adsorption onto the surface of clay particles provides an important energy source in the predominantly oligotrophic soil environment (Coleman et al., 2004). Soil texture has also been found to affect the susceptibility of bacteria to predation. Rutherford and Juma (1992) found that fine textured soil such as silty clay and clay loam supported a greater number of *Pseudomonas* in the presence of the protozoan predator *Acanthamoeba* than a coarser sandy loam soil. Additionally, a greater increase in the number of protozoa was observed in the case of the latter soil type. This suggests that fine textured soils offer bacteria a greater degree of protection against predation due to the availability of a larger proportion of protected micropores relative to coarse soil. Franz et al., (2008b) also demonstrated that survival of *E. coli* O157 in a loamy soil was primarily regulated by soil texture, and observed a positive association between time to decay and proportion of clay particles. In contrast, other work has shown that OM is more influential than texture in determining pathogen survival (Cools et al., 2001; Lang and Smith, 2007). Nonetheless, it is evident that soil texture has important implications for the survival of microorganisms in soil.

The effect of pH on pathogen survival in soil has been well documented. Typically, the survival of enteric bacteria is reduced at low pH (Gerba et al., 1975), and tends to increase when approaching a neutral to alkaline state (Sjogren, 1994). A pH of between 6 and 7 has been identified as an optimum for survival (Reddy et al., 1981). However, some pathogenic *E. coli* strains appear to be well adapted to acidic conditions.

Lin et al., (1996) has suggested that there may be an association between virulence of pathogenic *E. coli* and its ability to survive in the highly acidic gut environment within the host. Therefore, pathogenic strains may be particularly capable of surviving for extended periods in acidic soils following environmental release. This argument is strengthened by the observations of Franz et al., (2008b) who reported an inverse relationship between pH and *E. coli* O157:H7 survival. This demonstrates that effect of pH on pathogen survival is complex and can be strain dependent.

1.6.1.2. Soil structure

Soil structure is another factor that potentially affects the survival of faecally-derived pathogens. Structure plays an important role in determining soil hydrology. It can influence soil pore distribution, water retention and conductivity, and any changes to soil structure can greatly alter these inherent hydraulic processes (Kutílek, 2004). Aggregate formation is initiated by a combination of disturbance and freeze-thaw cycles. Clays are also fragmented by swelling and contraction as a result of wet-dry cycles. Exudates and secretions of extracellular polysaccharides by plant roots and microbes facilitate cohesion of soil aggregates, and structure is further stabilised by networks of plant roots and fungal hyphae which extend through the soil at a range of spatial scales, reviewed in Morales et al., (2010). Burrowing organisms, such as earthworms, distribute OM through the soil profile. This activity results in the creation of biopores, thereby generating habitat space for other soil microorganisms and preferential pathways for the transport of water, gases, dissolved substances and nutrients (Bardgett, 2005). These preferential flow paths have been described as ‘biological hot-spots’, as they are associated with increased microbial biomass and biological activity (Morales et al., 2010; Bundt et al., 2001). Therefore, soil structure will largely determine the spatial distribution of introduced pathogens.

Additionally, the extent of physical protection conferred to the pathogen will be determined by the availability of microsites. Survival time may be lengthened if the pathogen finds its way to a protective microsite, which may restrict the movement of larger predatory organisms. Vargas and Hattori (1986) investigated the survival of *Aerobacter aerogenes* in the presence of the predatory protozoan *Colpoda* spp. This

study divided the soil aggregate into inner and outer zones of 2.5 µm and 34 µm respectively, based on the characteristic moisture release curve. It was shown that the smaller prey were capable of accessing the inner zone of the aggregate, whereas the larger predator was restricted to the outer zones. A study by Postma et al., (1989) showed that proliferation of *Rhizobium leguminosarum* was more likely following inoculation into soils with low initial moisture content, and that survival declined as moisture content increased. These findings were attributed to variable distribution of inoculant in the matrix based on microsite availability. Similarly, Wright et al., (1995) demonstrated the importance of structure in soil predator-prey relationships. This study achieved prescribed distribution of *Pseudomonas fluorescens* in small, intermediate and large sized pores of less than 6 µm, 6-30 µm and 30-60 µm neck diameter, respectively, by manipulating soil water status and matric potential, with reference to the soil-specific moisture-release-characteristic curve. It was shown that, in the presence of soil ciliate *Colpoda steinii*, cell concentrations of *P. fluorescens* declined at a greater rate in intermediate and large pores, while those in small pores remained viable for a longer time period. This was attributed to the inaccessibility of the small pores and consequent inability of the protozoan grazer to access its prey effectively.

1.6.1.3. Nutrient and oxygen availability

The presence of easily-degradable organic substrates may also influence pathogen persistence in soil. For example, it was shown that the addition of glucose at 1.25 mg g^{-1} of soil enhanced *E. coli* survival as compared to glucose addition of 0.125 mg g^{-1} of soil, resulting in death rates of 0.12 day^{-1} and 0.24 day^{-1}, respectively (Habteselassie et al., 2008). Also, the lower glucose concentration promoted greater *E. coli* decline when as compared to a control treatment which received no glucose, suggesting that nutrient input below a specific threshold level may be detrimental to pathogen survival in soil. This result was attributed to increased *E. coli* activity stimulated by low glucose addition; however growth could not be sustained due to rapid nutrient depletion by the more active *E. coli* population, and consequently a higher level of cell death was observed.

Pathogen persistence can also be influenced by the nutrient status of soil organic amendments. The use of slurry and artificial fertiliser tends to favour the rapid growth of R-strategists such as *E. coli* O157, due to the low C/N ratio and rapid release of nutrients to the soil. However, by using organic manures with a high C/N ratio and slower nutrient release, fast growing bacteria, which include pathogen species, will be excluded by slow-growing K-strategists, thereby regulating pathogen populations in the soil (Franz et al., 2008b).

The presence of a rhizosphere may also play an important role in sustaining introduced pathogens as compared to bulk soil due to the release of nutrient-rich root exudates. Gagliardi and Karns (2002) found that *E. coli* inoculated into fallow soil microcosms survived for 25-41 days. However it could persist for up to 96 days on rye roots, demonstrating the ability of a rye rhizosphere to support *E. coli* populations. Similar persistence in the presence of rye was witnessed by Sjogren et al., (1995), who could isolate *E. coli* from plots planted with rye-grass 13 years after inoculation. Alfalfa roots also enhanced pathogen survival however other legumes, namely crimson clover and hairy vetch, did not, indicating that rhizosphere effects may be plant species-specific (Gagliardi and Karns, 2002). Also, Williams et al (2007b) reported that the presence of a maize rhizosphere had no significant impact on *E. coli* O157:H7 survival in soil amended with cattle slurry and ovine stomach content, and similar rates of decline were observed in both planted and unplanted soil samples. In this case, rhizosphere effects were deemed of lesser importance than other abiotic and biotic factors, such as the nature of the organic amendment, soil type, temperature and moisture content, and competition with soil microflora.

Oxygen availability also plays a key role in determining pathogen survival. A number of human pathogens, including *E. coli*, *Salmonella*, *Listeria* and *Cryptosporidium* are facultative anaerobes. This means that they survive best when O_2 is limited, but they are also capable of adjusting their metabolism to grow aerobically. Semenov et al., (2010) investigated survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in cow manure and slurry under both aerobic and anaerobic conditions. It was found that *E. coli* O157:H7 persistence was enhanced when O_2 was limited, and survival time increased from 2 weeks under aerobic conditions to 6 months under anaerobic conditions at 16^{o}C when moisture was constant. This was attributed to the

effect of O_2 on chemical reactions within the manure and slurry, and consequent changes in microbial community composition. However, there was no significant effect of O_2 availability on *Salmonella* serovar Typhimurium survival. It was suggested that these organisms react differently to biological and chemical changes stimulated by O_2, and that *S.* Typhimurium is metabolically more flexible to survive in a range of O_2 conditions than *E. coli*.

In contrast, *Campylobacter* is microaerophilic and requires decreased O_2 and increased CO_2 relative to atmospheric conditions for growth. The optimal gaseous proportions for cultivation have been calculated at 85% N, 10% CO_2 and 5% O_2 (Pennie et al., 1984). *Clostridium* is an obligate anaerobe and thus requires an oxygen-free environment for growth. However *Clostridium perfingens*, which is a common zoonotic pathogen, can survive in the presence of oxygen and a number of genes can be switched on to cope with this oxidative stress (Briolat and Reysset, 2002).

Therefore, it is clear that O_2 requirement is highly pathogen-specific and variation in the level of O_2 can greatly alter the physical and chemical environment that the pathogen encounters.

1.6.1.4. Moisture and temperature

Many studies have been carried out to investigate the effect of moisture and temperature on pathogen survival, and it has been shown that pathogen persistence is typically associated with cool, moist conditions (Cools et al., 2001). Temperature extremes resulting in freeze-thaw cycles and desiccation can reduce pathogen viability (Rosen, 2000). Pathogens are also negatively affected by exposure to UV irradiation, which can damage cell structure when exposed on the soil surface (Hutchison et al., 2004b).

The inactivation rate of *Cryptosporidium parvum* oocysts in soil and animal waste was more rapid between temperatures of 35^{o}C and 50^{o}C and with decreasing soil water potential (Jenkins et al., 1999). *E. coli* survival was enhanced at 6^{o}C as compared to 23^{o}C, and exposure to fluctuating temperatures between 6^{o}C and 23^{o}C, representative of environmental conditions, resulted in intermediate survival (Habteselassie et al., 2008). Cools et al., (2001) determined *E. coli* cell concentrations in a loamy soil as a function of temperature and moisture. The limit of detection was reached following 80

days of incubation at 5°C and 100% field capacity; however this limit was observed after only 38 days at 25°C and 60% field capacity. Survival of *E. coli* O157:H7 in bovine faeces was assessed at loading rates of 10^3 and 10^5 CFU g^{-1}, and was found to range from 42 to 49 days, 49 to 56 days and 63 to 70 days at 37°C, 22°C and 5°C respectively (Wang et al., 1996). Topp et al., (2003) reported a more rapid decline in viable *E. coli* populations at 30°C as compared to 4°C in clay and sandy loam soils inoculated with pig manure slurry. Also, Ogden et al., (2001) found that the decline in susceptible sub-populations of *E. coli*, which represented more than 95% of the total *E. coli* population in a slurry sample, was greater at 15°C as compared to 6°C and at low soil moisture. However, the difference in survival rates between temperature and moisture treatments among the remaining 5% of resistant sub-populations was not statistically significant, demonstrating that a proportion of the population may be able to withstand unfavourable environmental conditions.

According to Arrus et al., (2006), *Salmonella* exhibits a similar survival trend to that of *E. coli* under different moisture and temperature conditions. Following manure application, *Salmonella* concentrations in soil fell close to the limit of detection 95 to 100 days after incubation under low temperature (4°C) and high moisture (80% field capacity) conditions. However, the limit of detection was observed after only 30 to 75 days incubation under high temperature (25°C) and lower moisture (60% field capacity) conditions, further demonstrating the optimal regimes in terms of survival. Other work has shown that in manure, survival of Salmonella is superior to that of *E. coli* (Franz et al., 2005), and that the negative affect of increasing temperature is more pronounced for *E. coli* (Semenov et al., 2007). Interestingly, this research also showed that these pathogens could survive better when maintained at static temperatures as opposed to fluctuating temperatures that would likely occur in the environment. This suggests that risk assessments which model pathogen survival based on constant temperature values may overestimate the transmission potential (Semenov et al., 2007).

Repeated freeze-thaw cycles, exposure to UV irradiation and desiccation can also reduce viability (Rosen, 2000). Persistent freezing has been shown to enhance survival of *E. coli* O157:H7 in soil. In a lab-based study, viable cells were recovered from 37% of thawed soil samples following 500 days at -20°C, with recoveries of 44% observed for soils with a higher clay fraction (Gagliardi and Karns, 2002). Habteselassie

et al., (2008) found that bacterial survival in soil subjected to freezing was dependent on the initial water potential of the soil, and that *E. coli* cells in wet soils declined more rapidly than in dry soils after thawing. Incorporation of slurry and manure directly after spreading resulted in a slower rate of decline in laboratory-cultured *E. coli* O157, *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. as compared to the decline observed when left on the soil surface for one week prior to incorporation (Hutchison et al., 2004b). This was attributed to the buffering effect and lack of environmental control in the sub-surface as compared to the surface of the soil. Pathogens were not exposed to rapid drying, UV irradiation and extreme temperature fluctuations when incorporated, and therefore survival was enhanced.

1.6.1.5. Land management

Land management can exert a large influence on pathogen survival due to complex interactions with the physico-chemical and biological environment. Franz et al., (2008b) investigated the survival of *E. coli* O157:H7 in a variety of manure-amended soils under conventional and organic management. No difference in survival was observed between management types, likely due to similar soil composition. However, this study used a multiple regression approach to show that dissolved organic C and N, and levels of easily available carbon substrate per unit of soil biomass were the best predictors of *E. coli* O157:H7 survival; inactivation was associated with low nutrient availability. It was therefore suggested that promoting an oligotrophic soil environment could be an effective management tool to minimise the risk of *E. coli* transmission. This could be achieved by reducing the input of mineral fertilisers which constitute a readily available source of N, and increasing the input of complex organic amendments with a high C/N ratio (Franz et al., 2008b). However, this may ultimately have negative consequences for the productivity of the system. Nyberg et al., (2010) investigated the effect of manure type on the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium in an outdoor lysimeter study. Survival ranged from 15 to 90 days for urine and poultry manure, respectively. Greater persistence in the presence of poultry manure was attributed to higher OM content, which may have buffered the pathogens from the

oligotrophic soil environment, and provided greater surface area to escape predation and competition from native microorganisms.

Pathogen survival can also be influenced by cropping and tillage practices due to plant species-specific rhizosphere effects. The potential of the rhizosphere to harbour opportunistic pathogens is reviewed in Berg et al., (2005). Cooley et al., (2006) found that the survival of *E. coli* O157:H7 in the rhizosphere of lettuce was reduced due to the presence of *Enterobacter asburiae*. This indigenous microorganism utilises the same carbon sources as *E. coli*; however it could compete more effectively and thus prevented *E. coli* from becoming established in the rhizosphere. It would therefore be desirable to promote agricultural practices that could exploit this competitive exclusion mechanism, and foster such competitive bacteria in rhizosphere soil to reduce the pathogen risk associated with crop production. In contrast, it has also been shown that *E. coli* O157:H7 survival is not significantly affected by the presence of a maize rhizosphere when livestock manure has been applied; in this instance, manure type is more influential (Williams et al., 2007b).

1.6.1.6. Abiotic inactivation of viruses

The persistence of viruses in soil is equally pertinent in light of their inherent infectivity and their involvement in interactions with higher organisms that may be pathogenic in nature. Inactivation of viruses in soil is related to the prevailing abiotic conditions. Hurst et al., (1980) carried out a study to investigate the factors affecting inactivation of various strains of coxsackievirus, echovirus, poliovirus, rotavirus and bacteriophage in soil. Inactivation increased with increasing soil temperature and decreasing adsorption to soil particles. Virus survival tended to decrease with increasing soil moisture content until soil saturation point, and this trend was attributed to the influence of soil moisture on microbial activity and strength of virus adsorption to soil. Decreasing pH and increasing aluminium resulted in virus persistence as both of these parameters enhanced virus adsorption to the soil. Additionally, the availability of free phosphorus anions in soil encouraged virus desorption, and consequently, inactivation.

Zhao et al., (2008) investigated the effect of soil type, soil moisture content and the presence of indigenous microbes on MS2 bacteriophage inactivation. This study

found that the concentration of metal oxides such as Fe and Al in the soil was an important determinant of virus adsorption. Electrostatic interaction between these metal oxides and the virus resulted in loss of infectivity and inactivation. In metal-oxide rich soils, virus inactivation was associated with decreasing moisture content and sterilisation, both of which increased adsorption to soil particles. Zhao et al., (2008) concluded that in this case, the interaction between the virus and metal oxides was more influential than the presence of indigenous microorganisms.

Somatic coliphages were detected in soil 143 days following the application of coliphage-spiked manure. This demonstrates the longevity of enteric viruses in the soil environment. It also suggests that somatic coliphages may provide a more accurate representation of viral pathogen contamination than traditional bacterial indicators, which declined to reference levels 4 days after manure application. (Gessel et al., 2004)

1.6.2. Biotic considerations

In addition to the abiotic factors mentioned above, the biology of both the pathogen and the soil environment can affect survival. Pathogen-specific factors include physiological status and inoculum concentration, reviewed in van Veen et al., (1997). In addition, interactions between the indigenous microbial community and introduced pathogens must also be taken into account. To date, the role of biology in pathogen suppression has been neglected, arguably due to the fact that the tools required for investigating biological associations within in the soil matrix have been limited. However, with the advent of molecular microbial ecology and rapid technological development in this field, the relatively unexplored 'black box' of soil biology has become more accessible (Tiedje et al., 1999, Hirsch et al., 2010).

Interactions between pathogens and the native microbiota were first investigated in pioneering studies e.g. Waksman and Woodruff, 1940; Garcia and McKay, 1970. These interactions are governed by various mechanisms, such as predation, competitive exclusion based on substrate utilisation and niche space occupation. Antagonism is a mechanism of growth inhibition and by definition includes competition, antibiosis, amensilism and predation (Lartey, 2006). The main soil antagonists include protozoa, namely ciliates, flagellates, testate and naked amoeba and also bacteriovorous nematode

populations which can display specialized grazing on bacterial prey. Also important to consider are bacteria and fungi in both manure and soil matrices which may prove antagonistic to pathogenic bacteria and viruses, contributing to their decline.

The term ‘biodiversity’ has been a key theme in soil ecology in recent years, and although it is generally accepted that maintaining biodiversity is important, its functional significance remains somewhat ambiguous (e.g. Bardgett et al., 2005). Biodiversity is typically described by genetic complexity at species level, and encompasses both the number of species present and relative abundance of such species, termed richness and evenness, respectively. However, due to the enormous diversity witnessed in soil, comprising billions of bacteria, thousands of nematodes and protozoa, hundreds of macroorganisms and a variety of extensive fungal hyphae and plant root systems (Ritz, 2005), it becomes more difficult to describe or consider using the traditional species approach. Microbial diversity can also be described with respect to functional attributes and trophic interactions of the communities present. In addition, microbial community structure must also be taken into account when considering soil biological composition. This is described as the arrangement of the microbial cohort within the soil matrix. A change in the community may not necessarily be reflected by a corresponding change in diversity, as fluctuations within one group of organisms may be offset by fluctuations within another (Hartmann and Widmer, 2006). Therefore, in order to provide a comprehensive assessment of soil biology, it is important to use methods at varying levels of organization, ranging from genetic and molecular to community function and structure (Torsvik and Øvreås, 2002; Hirsch et al., 2010).

1.6.2.1. Origins of soil biodiversity

It has been found that microorganisms introduced into soil decline more rapidly when other indigenous microbes are present (Jiang et al., 2002), particularly if the indigenous microbial community is diverse (Ibekwe et al., 2010). Soil is a heterogeneous, complex and dynamic environment and therefore a wide variety of niches are available for occupation both temporally and spatially. The ‘soil microbiostasis’ concept, which describes the susceptibility of an ecosystem to invasion, states that the number of

ecological niches available to introduced organisms is reduced as a community becomes more biologically diverse. This is because there are a greater number of individual species present in the matrix, with distinct optimal conditions for survival (Ho and Ko, 1985; van Elsas et al., 2002). A primary driver of biodiversity in soil is the extent of heterogeneity with respect to both soil structure and nutrient resource composition (Bardgett, 2005). If the matrix is heterogeneous and receives complex organic inputs, there will be a variety of physical habitats and substrate available for utilisation by metabolically diverse microbial communities. This will result in a relatively uniform community structure exploiting many of the available ecological niches. However, if a matrix is homogeneous in terms of physico-chemical composition, then a selection of competitive microbes may become dominant. If the soil matrix is heterogeneous and lacks biological diversity, niches may remain unexploited, providing opportunity for proliferation of pathogens and microbes introduced via landspreading.

Spatial isolation is also an important determinant of soil biodiversity. Zhou et al., (2002) compared diversity patterns between surface and sub-surface soils where carbon was limited, and observed a uniform, non-competitive and dominant competitive community structure for each soil type, respectively. As the presence of water is transient on the soil surface, transport of both nutrients and microbes is limited temporally and thus in the absence of resource abundance and heterogeneity, this spatial differentiation restricts growth and dominance of microbial communities and promotes uniform microbial distribution. Zhou et al., (2002) also found that a non-competitive diversity pattern prevailed in both surface and sub-surface soils where unlimited and varied resources were available, indicating that substrate and lack of competition were primarily influencing diversity in this case. The importance of soil organic carbon stores in maintaining diversity was also demonstrated by Degens et al., (2000), who found a significant linear relationship between catabolic evenness of the microbial community and organic C. Therefore, different organic management regimes, which will determine the quantity and quality of soil OM, will have site-specific impacts on soil microbial biomass and community structure (Lejon et al., 2007). Additionally, diversity has been related to land-use intensity, fertilizer application and, to a lesser extent, seasonality (Jangid et al., 2008). Intermediate disturbance tends to promote diversification of the microbial community. Higher diversity was witnessed in pasture soil as compared to

forest and crop-land soil which represented low and high disturbance respectively (Jangid et al., 2008). A similar occurrence was noted by Upchurch et al., (2008). This low diversity of forest soil was attributed to dominance of specific microbial groups under stable environmental conditions. Of all edaphic factors that influence microbial diversity, community structure and catabolic functioning, soil pH and texture have been identified as most significant for bacterial and fungal populations, respectively (Wakelin et al., 2008). Fluctuating environmental conditions and the resultant diversity of niches created can also maintain biological diversity in the soil habitat (Ritz, 2005).

1.6.2.2. Consequences of soil biodiversity on enteropathogen survival

The interactions between biodiversity, niche availability and pathogen survival can be determined in controlled laboratory-based microcosm experiments, in which samples of varying degrees of diversity are established. Studies in such circumstances generally show that survival tends to be compromised in samples approaching a non-sterile state, presumably due to the antagonistic effect of resident microflora. For example, it was found that survival of a lux-marked strain of *E. coli* O157:H7 was significantly influenced by the extent of soil biodiversity (van Elsas et al., 2007). Different levels of diversity were established by chloroform fumigation, and survival of *E. coli* was assessed 60 days following inoculation using a suite of microbiological methods, including soil microbial carbon, soil respiration and enzyme analysis, comparison of total and cultivable bacterial counts, phospholipid fatty acid (PLFA) analysis and deoxyribonucleic acid (DNA) fingerprinting, thus providing a comprehensive picture of shifting microbial community composition and its impact on introduced *E. coli* populations over time. *E. coli* survival was enhanced in soils which had received the highest fumigation level as compared to intermediate and low levels, and this was supported by both culture-based and culture-independent analyses. It was concluded that persistence of the pathogen in soils exposed to intensive fumigation was a consequence of decreased community complexity (van Elsas et al., 2007). Matos et al., (2005) used a dilution-extinction approach to create high, intermediate, artificially constructed and low levels of diversity in the wheat rhizosphere, and investigated the relationship between diversity and invasibility of *Pseudomonas aeruginosa*. *P.*

aeruginosa cells were enumerated for each treatment following a 14 day growth period, and a sequential increase was observed from high to low diversity, demonstrating an inverse relationship between these parameters. A study by Jiang et al., (2002) found a more rapid decline in *E. coli* concentration in manure-amended non-autoclaved soil as compared to manure-amended autoclaved soil at both 15^{o}C and 21^{o}C, demonstrating that indigenous populations contribute to pathogen inactivation. Prolonged survival was observed at 5^{o}C, which was likely due to the lack of competitiveness of antagonistic microbes at lower temperatures. The same study also showed that decline was more rapid in autoclaved soil with increasing manure loading rates. Survival was compromised in concentrated manure/soil formulations, and this was attributed to greater manure microbe populations and nutrient availability found in the more concentrated mix.

1.6.2.3. Predation

The interaction between indigenous predatory microbes and introduced pathogens is an important determinant of pathogen survival. Acea et al., (1988) studied the effect of predation, lysis and amensilism on exogenous bacteria in soil. It was found that bacteria survived and grew well in sterile soil, indicating that biological factors are important determinants of bacterial success. Protozoan grazing was predominantly implicated in the decline observed in non-sterile soil, mostly due to the increase in predator populations associated with the death of test species. This research also showed that survival was prolonged following treatment with a eukaryotic inhibitor. Lytic- and antibiotic-producing microbes were also implicated in the death of some test species; however the association under natural field conditions may differ from that found in the lab. Other work has indicated that protozoan grazing can actually have a protective effect on microflora that can evade digestion (Barker et al., 1999). For example, the *Acanthamoeba polyphaga* has been shown to engulf *E. coli* O157 and store the cells in food vacuoles which may enhance the resistance and resilience of the pathogen in the environment. This interaction can also prevent the decay of *E. coli* O157 induced by typical sanitation measures such as the application of sodium hypochlorite to crop surfaces. The pathogen is capable of emerging from these vacuoles either within the

predator or following excretion to the environment (Barker et al., 1999). Also, a study carried out by Gourabathini et al., (2008) showed that grazing of *E. coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* by different ciliates isolated from leaves of lettuce and spinach resulted in varying degrees of protection. *Glaucoma* spp. released food vacuoles containing a high proportion of viable *E. coli* and *S. enterica* and a considerably lower proportion of *L. monocytogenes*. *Tetrahymena* released all but the latter, and *Colpoda stenii* and *Acanthamoeba palestinesis* did not produce vacuoles following ingestion of any of the pathogens employed. Thus it has been suggested that although certain grazing interactions may prove antagonistic to the pathogen, some might also prolong their survival in the environment.

In addition to protozoa, bacteria feeding nematodes are abundant in soil ecosystems and can ingest 10^5-10^6 bacterial cells per day (Venette and Ferris, 1998). Therefore it is not surprising that nematodes can shape microbial community structure, and can suppress or enhance activity, depending on the extent of grazing pressure. The effect exerted on the microbial community is dependent on the species of nematode present. For example, Djigal et al., (2004) found that species of *Cephalopidae* altered the dominant bacterial populations present over time, as reflected by the intensity of denaturing gradient gel electrophoresis (DGGE) bands, but did not alter composition. This was in contrast to other studies, for example, Griffiths (1994), who observed distinct shifts in community structure following the addition of bacteriovorous nematodes, namely *Coactadera cystilarva* and *Panagrolaimus* spp. to loamy soil and sand. These shifts were detected on a functional basis by community level physiological profiling (CLPP) and PLFA, and genetic variations were detected using DNA extraction and subsequent molecular analysis. The disagreement between these studies was attributed to the phylogenetic similarity between the species of *Cephalopidae* used by Djigal et al., (2004). Therefore the impact of nematode populations on community structure will be greatest between families. De Mesel et al., (2004), found that in microcosm experiments using decaying cordgrass as substrate, opportunistic nematodes, e.g. *Rhabditida* increased at a greater rate than selective feeders, e.g. *Monhysterid*, thereby exerting a greater pressure on the microbial population. This overgrazing simplified the microbial community structure and resulted in lower bacterial diversity over the 65 day incubation period, which was reflected by microbial respiration rates

and proteolytic enzyme production. Contrastingly, greater diversity was observed for slower growing selective *Monhysterid* species, which exerted intermediate grazing pressure. This demonstrates that the rate of grazing can influence microbial diversity in soil, and that intermediate pressure tends to favour the establishment of greater diversity. Selective grazing habits of various nematode species may also determine bacterial community structure. It was found that species of *Cephalopidae* did not multiply successfully when fed on *Actinomyces* spp. and Gram-positive *Arthrobacter* spp. This was attributed to the escape capability endowed by the buccal cavity of large flagellate bacterial cells, and also to the thick cell wall of Gram-positive bacteria, which is, in many cases, inedible and cannot be digested by the nematode (Blanc et al., 2006). Also, some selective grazers have been shown to respond to species-specific chemical signals produced by bacteria, and this may infer preference (De Mesel et al., 2004). Microorganisms may also respond to nematode activity in soil, such as bioturbation and mucous production. Bioturbation can facilitate the transport of oxygen and nutrients through the soil matrix, thus altering microhabitats of the bacterial community. Additionally, the establishment of microbial colonies has been observed in the nematode mucous trail, which appears to sustain microbial growth (De Mesel et al., 2004).

1.6.2.4. Competition

Decline in inoculant populations to soils has been observed in the absence of predation, and this has been attributed to competition between introduced and indigenous microflora. In a sample devoid of protozoan species, inactivation was still 10^4 times greater than that observed in a fully sterile sample (Recorbet et al., 1992). Also, Byappanahalli and Fujioka (2004) showed that the ratio between minor populations of faecal bacteria and indigenous microflora in a Hawaiian soil is approximately $1{:}10^{10}$, demonstrating potential for fierce resource competition in the soil environment. Growth of the faecal population was observed following addition of substrate to the soil, indicating that the limiting influence of competition may be minimized when nutrients are in excess and conditions are suitable for multiplication. Postma et al., (1990) investigated the importance of microniche availability for the establishment of inoculated *Rhizobium leguminosarum* biovar Trifolii, and the regulation of competitive

and predatory interactions with the indigenous microbial community. Sterile loamy sand was simultaneously recolonised by 80 bacterial strains, which were isolated and cultured from non-sterile loamy sand, and these were assumed to act as soil competitors. The flagellate *Bodo saltans* was cultured on rhizobial cells with frequent dilution to prevent colonisation by other bacteria and employed as a predator in recolonised soil. When rhizobia were introduced to soil containing the predator or competitor only, a variable reduction in cell number was observed which was dependent on soil type (Postma et al., 1990). A greater proportion of rhizobial cells were associated with soil particles and micropores when introduced with the flagellate alone, and this proportion was reduced in the presence of competitors. When introduced to soil with both predator and competitor, a synergistic interaction occurred resulting in a drastic reduction in rhizobial cell concentration. This synergism was explained by the fact that when inoculated into soil, a greater proportion of both indigenous and non-indigenous cells became concentrated in micropores with time, due to predation on the outer part of soil aggregates and consequently, the success of introduced cells in such micropores was dependent on their ability to compete effectively with indigenous microflora (Postma et al., 1990). Topp et al., (2003) observed that the dominant *E. coli* strains detected in pig manure slurry were distinctly different from those detected in soil inoculated with pig manure slurry after 6 days incubation, as revealed by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) fingerprinting. This demonstrates that the growth and survival of *E. coli* in soil is strain-dependent, and establishment will be determined by competitive competency.

It may be argued that the indigenous microflora are better equipped to survive in the oligotrophic environment of bulk soil as compared to introduced pathogens, as these populations have adapted to prevailing conditions over time. Land application of organic waste may create a famine-to-feast situation in the soil due to enhanced substrate availability. This could stimulate an increase in microbial biomass, and survival of introduced pathogens will be determined by proficiency in utilising this substrate as compared to the indigenous community. Therefore, the role of competition in pathogen survival must also be elucidated

1.6.2.5. Antimicrobials

Many soil microorganisms naturally produce antimicrobial compounds that can inhibit pathogen survival. These compounds are metabolically expensive to produce but typically confer a competitive advantage to the parent organism. For example, species of *Pseudomonas* and *Burkholderia* are capable of producing pyrrolnitrin, which is effective against human pathogenic bacteria and many economically important species of fungi such as *Rhizoctonia solani* and *Verticillium dahliae* (reviewed in (Raaijmakers et al., 2002)). Most of the research on in-situ antimicrobial activity has focused on biocontrol of plant pathogens. For example, Raaijmakers and Weller (1998) found that under experimental conditions, the antibiotic 2, 4-diacetylphloroglucinol (Phl) produced by a fluorescent *Pseudomonas* spp. was highly effective in reducing take-all decline caused by *Gaeumannomyces graminis* var. Tritici in a wheat crop. This antibiotic was also involved in reducing the incidence of black root rot in soils naturally suppressive to *Thielaviopsis basicola*, which improved the yield of tobacco (Ramette et al., 2003).

A study carried out by Takahashi et al., (2008) screened a suite of fungal species isolated from Brazilian soil for antibacterial activity against a range of pathogens including *E. coli*, *S. aureus*, *S.* Typhimurium, *S. pyogenes* and *L. monocytogenes*. Over half of the 200 fungal strains tested displayed antagonistic activity towards at least one pathogen. A review by Mazzola (2002) suggested that soil communities could be manipulated to promote the proliferation of known antagonists through specific cropping practices and other management techniques, with the aim of reducing the incidence of soil borne plant pathogens and resultant crop disease. This could potentially be extended to include a reduction in human pathogens.

1.6.2.6. Viral infection

Bacterial survival can also be influenced by viral infection. Phages are common soil viruses that typically infect bacteria in a process termed lysogeny, whereby the phage is incorporated into the bacterial chromosome. Alternatively a soil bacteriophage may exhibit lytic infection, whereby the host metabolism is redirected toward the creation of additional viruses, eventually causing the bacterial cell to burst open as a result of cell

lysis. Lysogeny is preferable in the soil environment, as phages can survive prolonged periods of host inactivity within the host bacterium (Kimura et al., 2008).

Keel et al., (2002) showed that the biocontrol activity of *Pseudomonas fluorescens* strain CHAO toward the fungal pathogen *Pythium ultinum* in a cucumber rhizosphere was severely compromised in the presence of the lytic phage, ΦGP100. This phage was capable of reducing the *Pseudomonas* population by over 100-fold, which was associated with an increase in phage concentration. Yamada et al., (2007) characterised four bacteriophages that utilised the various strains of the bacterial-wilt pathogen, *Ralstonia solanacearum*, as a host. This study found that infection by ΦRSA1 and ΦRSL1 reduced the incidence of bacterial wilt in tobacco plants, (*Nicotiana tabacum*), which was attributed to the wide host range of these phages. A decline in pathogen growth rate was observed following infection by a third phage ΦRSS1. However, this phage also altered the pathogenicity of one host strain (C319), and actually enhanced disease expression among tobacco plants.

The potential of bacteriophages for control of *E. coli* O157:H7 has been demonstrated by Kudva et al., (1999). Phages specific to the O157 antigen were isolated from bovine and ovine faecal samples and subsequently inoculated to lab cultures of *E. coli* O157:H7 to determine lysis efficiency. Three of the phages identified, namely KH1, KH4 and KH5, were used for further analysis. It was found that when the three phages were added simultaneously to the culture and incubated at 37^{o}C with aeration, complete lysis of the bacterial cells was achieved after 8 hours of infection. *E. coli* O157:H7 concentration was reduced by a factor of 10^{9} in the test culture as compared to the control, to which no phages were added. Additionally, non-O157 *E. coli* and non *E. coli* were not affected by phage activity. These results suggest that some bacteriophages may target O157 populations in the soil environment. Further, O157-specific phage isolates might be used to control O157 proliferation in the gastrointestinal tract of livestock (Kudva et al., 1999). Sheng et al., (2006) achieved a significant reduction in *E. coli* O157 in cattle ruminants following rectal application of phages SH1 and KH1; however the pathogen was not fully eradicated from the cattle following 7 days of treatment, and *E. coli* O157 was still detectable in 4 of 5 calves to which the phages had been administered. O'Flynn et al., (2004) also showed that a 'phage cocktail' comprised of lytic phages, e11/2, e4/1c and pp01, could effectively reduce *E. coli* O157:H7

concentrations on meat surfaces. Despite the emergence of a small proportion of bacterial mutants capable of phage resistance, the authors recommended the use of these phages for environmental biocontrol of the pathogen.

Such work suggests that the viral component in soil may play a significant and mostly neglected role in pathogen inactivation, through phage-host specific interactions.

1.6.2.7. Biotic virus inactivation

Virus inactivation may also be caused by the presence of bacteria, through production of proteases and other antagonistic mechanisms (Sobsey et al., 1980; Pell, 1997; Kimura et al., 2008). Davies et al., (2006) observed a more rapid decline in PRD1 bacteriophage in non-sterile loam soil as compared to loam soil sterilized by gamma irradiation. This biotic effect on virus survival was not detected in a clay loam, and it was postulated that the clay particles inferred protection from bacterial antagonism, thus prolonging PRD1 survival in the clay loam soil. A study by Deng and Cliver (1992) compared Poliovirus Type 1 inactivation rates following inoculation into both an autoclaved and unautoclaved mixture of septic tank waste and pig manure slurry at 25^{o}C and 37^{o}C. A 90% reduction in viral activity was achieved following 6.8 and 11.2 days at 25^{o}C and 1.3 and 3.9 days at 37^{o}C in autoclaved and unautoclaved mixed waste, respectively. The greater decline observed in the unautoclaved sample was attributed to the antiviral effect exerted by bacteria present in pig manure slurry, which are presumably more active at higher temperatures. Microorganisms are capable of producing metabolites and proteolytic enzymes which enhance inactivation, and can also utilize the virus capsid as substrate. This effect was demonstrated by the addition of proteolytic inhibitors to the mixed waste, which resulted in inhibition of virus inactivation. It has also been shown that viral persistence is enhanced within deeper soil layers as compared to the humic zone where the majority of microbial activity takes place (Olszewska et al., 2008). Further, Hurst et al., (1980) compared Poliovirus inactivation rates under both aerobic and anaerobic conditions and found that decline was greatest in the presence of oxygen, thus implicating aerobic microorganisms in this process. Davies et al., (2006) investigated the persistence of PR1 bacteriophage and Adenovirus 2 in loam and clay loam soils at 4, 20 and 35^{o}C. Inactivation was greater in both soils at 35^{o}C, and

decreased with decreasing temperature, reflecting the effect of microbial activity on virus persistence. The effect of microbial enzymatic activity on virus survival is also virus-type dependant (Nasser et al., 2002). This study investigated the effect of microbial enzymes on the survival of different viruses, namely bacteriophage MS2, Cox-A9, Poliovirus Type 1 and Hepatitis A, in soil. Firstly, the viruses were exposed to varying concentrations of the proteolytic enzyme, protease pronase. A 90 % inactivation of Cox-A9 was observed following exposure, irrespective of enzyme concentration. However, MS2 was not affected by exposure and the effect on Poliovirus Type 1 and Hepatitis A was minimal. Additionally, virus inactivation in the presence of *Pseudomonas aeruginosa*, which can produce a variety of extracellular enzymes, was determined. *P. aeruginosa* activity resulted in a 99 % inactivation of both Cox-A9 and Hepatitis A; however MS2 and Poliovirus Type 1 were not affected. Nasser et al., (2002) concluded that viral composition may influence susceptibility to bacterial enzymes.

1.7. Suppressive soils

Some soils may possess an inherent suppressive capacity towards invading pathogens, which is contingent on interactions between the natural community of the soil and the pathogen in question. Suppression is also inextricably linked to abiotic soil characteristics, which will partly determine microbial activity. This phenomenon has been predominantly investigated with respect to plant pathogens (phytopathogens), to elucidate the factors that govern the incidence of crop diseases such as Fusarium wilt, take-all of wheat and Rhizoctonia, reviewed in Mazzola (2002). For example, Shiomi et al., (1999) compared two Japanese soils taken from a tomato rhizosphere for suppression of bacterial wilt caused by *Ralstonia solanacearum*. One of the soils, termed Mutsumi, was known to be suppressive toward the pathogen, whereas the second, termed Yamadi, was conducive. In order to demonstrate a causal relationship between suppression and microbial community structure, three treatments were established. Firstly, the Mutsumi soil was exposed to chloroform fumigation to reduce community complexity. Secondly, the Mutsumi soil was sterilized by autoclaving and mixed with non-sterile Mutsumi soil. Finally, the Mutsumi soil was again sterilized and

mixed with non-sterile Yamadi soil. The results of this study showed the highest incidence of *R. solanacearum* in the chloroform fumigated soil, and that the Mutsumi mix was significantly more suppressive toward the pathogen than the Yamadi mix. The microbial communities of both soils were subsequently analyzed, and it was found that the suppressive Mutsumi soil was characterized by high microbial diversity, whereas the Yamadi soil was relatively simple in comparison. Therefore, the natural suppression witnessed in the Mutsumi soil was attributed to a more diverse soil community, and competition between the pathogen and the resident rhizosphere microflora (Shiomi et al., 1999).

Similarly, Toyota and Kimura (2000) investigated the relationship between suppression and community structure in a series of laboratory-based experiments. In this study, sterile soils were inoculated with various strains of fungal and bacterial isolates, termed the 'priorcolonists', and subsequently inoculated with the pathogen of interest, in this case, *R. solanacearum* YU1if43lux. Suppression toward the pathogen was significantly greater in the treated soils than the control, to which no 'priorcolonists' had previously been added. Additionally, bacteria were implicated as the primary agents of suppression. Further investigation of antagonism exerted by individual strains toward *R. solanacearum* revealed that *R. solanacearum* NPS1, *R. pickettii* MSP3 and *P.fluorescens* MelRC2 were particularly effective. Mechanisms employed by these strains were associated with relatedness to *R. solanacearum* and rhizosphere competence. Other related work carried out by Toyota et al., (2000) found that the suppressive effect generated in soil was largely determined by the species of 'priorcolonist'. This study showed that the establishment of Type III *R. solanacearum* was particularly limited where other Type III strains had previously colonized the soil. This effect was not as pronounced when Type I and II strains were used as 'priorcolonists', and very little suppression was observed following prior colonization by strains belonging to other species. It was postulated by Toyota et al., (2000) that the degree of suppression observed was related to the ecological similarity to the Type III pathogen, and the consequent overlapping requirements for nutrients and physical space within the soil matrix.

Agricultural management regimes can impact on the natural capability of soils to suppress plant pathogens. It was demonstrated that the community structure of

Pseudomonas spp. varied between soils from permanent grassland, short-term arable and long-term arable land (Garbeva et al., 2004a). Permanent grassland supported the greatest total numbers of *Pseudomonas* as compared to arable soils. Additionally, more *Pseudomonas* exerting both direct antagonism, and antibiosis or chitinolytic activity toward the potato pathogen, *Rhizoctonia solani*, were isolated from permanent grassland. The enhanced suppression of *R. solani* in permanent grassland soil was attributed to the presence of grass roots, which presumably created conditions favorable for the proliferation of antagonistic *Pseudomonas*. Similarly, van Elsas et al., (2002) investigated the relationship between total microbial diversity as influenced by cropping history and antagonism toward *R. solani*. Using both cultivation dependent and independent techniques, it was shown that bulk and rhizosphere soil from permanent grassland continuously supported a more diverse and even bacterial and fungal community structure as compared to bulk arable soil, and consequently proved more suppressive toward the potato pathogen. Based on such results, it is evident that it may be possible to manipulate land management to alter soil community structure and hence, transform a soil, previously conducive to pathogen invasion, to a soil capable of pathogen suppression (Garbeva et al., 2004b).

In addition to land-use, organic amendments can also be used to manipulate community structure by changing nutrient availability within the soil. Soil conditions can be optimized to reduce the availability of carbon, and thus aid pathogen suppression (Franz et al., 2008b). The incorporation of pig slurry to soil has been shown to significantly reduce the survival of *R. solanacearum* and consequently, protect the potato plant from infection (Gorissen and van Elsas, 2004). Although the cause of *R. solanacearum* decline was not conclusively evident from this study, it was hypothesized that slurry application caused a shift in community structure, detectable by molecular methods, which may have favored the growth of microbes that were specifically antagonistic toward *R. solanacearum*. Gotz and Samlla (1997) have previously found that pig slurry promoted the growth of *Pseudomonas putida*, which is known to possess suppressive characteristics, thus providing support for this hypothesis. The addition of compost has also shown potential to regulate phytopathogen establishment in soil. Schönfeld et al., (2003) found that both the persistence of *R. solanacearum* and the incidence of disease in experimental tomato plants were significantly reduced following

compost incorporation. Again, this was likely due to a shift in microbial community structure stimulated by the organic amendment.

The information that has been generated regarding the suppression of phytopathogens in recent years is relevant when considering potential management measures for controlling manure-derived pathogens which pose a threat to human health.

1.8. Livestock manure management

One potential way of limiting pathogen release to the environment via agricultural practice is by employing appropriate manure management techniques which manipulate both abiotic and biotic factors to reduce pathogen numbers in the manure matrix prior to slurry spreading. Management methods differ between liquid slurry and solid manure. Slurry is the dominant form of organic fertilizer produced in Ireland. In 2003, 37 million tones of manure were produced over the winter period; of this, 29.3 million tones was in the form of slurry and the remainder was solid manure (Hyde and Carton, 2005).

1.8.1. Physical treatment

Slurry is often treated by means of digestion, either aerobically or anaerobically. During aerobic digestion, oxygen is introduced mechanically, which encourages the growth of aerobic microorganisms. This removes obligate anaerobes from the slurry matrix, some of which are pathogenic (Burton, 1992). A large amount of heat is generated during aerobic digestion, and thus shorter slurry treatment times are required. Aerobic digestion can also contribute to odour control, which is of particular interest for pig slurry. However, there are disadvantages associated with this method, including high operational costs and management difficulties (Bernet and Béline, 2009). Therefore, anaerobic digestion is more commonly practiced. Anaerobic digestion is defined as the 'engineered methanogenic anaerobic decomposition of organic matter', and utilizes a range of both non-methanogenic and methanogenic anaerobic microorganisms to degrade OM and produce methane, which can be harnessed for energy (Côté et al., 2006). Anaerobic digestion can be carried out under psychrophilic (<20^{o}C), mesophilic (20-40^{o}C) or thermophilic (>40^{o}C) conditions (Mohaibes and Heinonen-Tanski, 2004).

Côté et al., (2006) showed that psychrophilic digestion of pig slurry at 20^{o}C for 20 days effectively eliminated indigenous *Salmonella*, *Cryptosporidium* and *Giradia*, and reduced coliforms and *E. coli* by 99.67-100%. Mesophilic digestion was shown to inactivate *Salmonella* Typhimurium following treatment for 10 days at 37^{o}C in cattle dung slurry (Gadre et al., 1986). Olsen and Larsen (1987) compared inactivation of vegetative pathogenic bacteria during mesophilic (35^{o}C) and thermophilic (53^{o}C) anaerobic digestion. A reduction of 90% within 1-3 days and 0.4-2 hours was observed, respectively. This also confirms that there is a positive association between pathogen inactivation rates and temperature. Thus, effective pathogen reduction can be achieved at lower temperatures if treatment time is extended.

Composting of solid manure is often employed, which relies on degradation of OM via aerobic processes that result in heat generation. Composting also results in an increase in ammonia and pH levels (Himathongkham and Riemann, 1999). As pathogen viability is compromised at high temperatures and pH, this process can achieve a high degree of manure sanitization (Heinonen-Tanski et al., 2006). However, there are limitations. Often, due to limited O_2 availability, significant temperature increases are only observed in the centre of the heap and pathogens may escape heat inactivation if located in the outer layers. Also, age is variable throughout the heap, and therefore recent manure may have a considerably greater proportion of active pathogens than older manure. Additionally storage of these heaps can sometimes cause problems due to on-farm space limitations (Heinonen-Tanski et al., 2006). Turning of compost heaps can resolve some of these issues. It results in a more even distribution of oxygen throughout the heap and may also reduce volume due to compaction of the manure. However, this process can be labour-intensive. Efficacy of composting heaps can be improved by insulating the lining of the heap with insulatory materials and installing grates at the base to encourage a good through-put of oxygen throughout the process (Heinonen-Tanski et al., 2006).

Grewal et al., (2007) compared the survival of *Listeria monocytogenes* and *Salmonella* Typhimurium in pig manure exposed to different treatments, namely thermophilic composting, pack composting, and liquid manure storage, both with and without aeration. This study found that the greatest pathogen reduction was achieved by thermophilic composting, during which a temperature of 55^{o}C was reached. Both *L.*

monocytogenes and *S.* Typhimurium concentrations had been reduced significantly on the third day of treatment; however despite this reduction, both pathogens were detectable after 56 days, indicating the potential for pathogen persistence during the composting process. Compost treatment at 25^{o}C also reduced pathogen concentration, albeit at a slower rate. Aerated liquid treatment promoted a greater decline than liquid treatment without aeration, and both pathogens were undetectable after 56 days in the former. This suggests that although composting achieves a rapid initial decline in pathogen concentration, liquid treatment can reduce pathogen persistence in pig manure (Grewal et al., 2007). Although significant pathogen reduction can be achieved by composting, there is potential for re-growth during subsequent storage of composted materials. For example, Sidhu et al., (2001) examined the re-growth of *Salmonella* Typhimurium in composted wastewater biosolids, and observed rapid colonization of sterile as compared to non-sterile compost. This indicates that antagonism exerted by microflora indigenous to the compost contributed to the suppression of pathogen re-growth, and the extent of suppression was related to storage time prior to land application. Therefore, long-term storage of compost may counteract pathogen inactivation accomplished during composting and allow re-growth due to nutrient depletion and reduced biological activity within composted materials over time.

Land disposal of stored livestock manure and slurry is also a means of treatment from a pathogens perspective, as environmental exposure to temperature extremes and UV irradiation can result in effective pathogen inactivation when spread on the surface of the soil (Rosen, 2000; Hutchison et al., 2004b; Semenov et al., 2009).

1.8.2. Chemical treatment

Chemical amendments can also be applied to livestock manure and slurry to reduce pathogen concentration. Lime products are most commonly applied, and are effective due to the resultant increase in pH, which causes pathogen inactivation (Heinonen-Tanski et al., 2006). Additionally, the application of urea has been described as potential means of chemical sanitization of manure. Vinnerås (2007) found that the conversion of urea to ammonia ultimately raised pH and gaseous ammonia concentration in faecal waste, thus achieving pathogen inactivation due to ammonia toxicity. This study found

that *Salmonella* Typhimurium and *Entereococcus faecalis*, which were seeded prior to treatment, were undetectable in faecal waste following 5 days and 20 days, respectively. Faecal coliforms were also undetectable after 5 days of treatment. Provided that this process takes place in a confined space, the liberated ammonia will be maintained in the waste, thus ensuring ongoing treatment. This mode of disinfection is also economically viable, as any cost incurred in urea application is offset by the production of ammonia-rich waste with enhanced fertilizer potential (Vinnerås, 2007). However, if ammonia emissions were to increase as a result of this type of treatment, this would ultimately reduce the agronomic value of the final manure product (Hao et al., 2001).

Plant essential oils also show potential for pathogen control. For example, Varel (2002) showed that pig slurry amended with carvacol achieved a 3_{log} reduction in faecal coliforms after 2 days. Faecal coliforms were undetectable in the slurry 14 days after treatment. Other work has shown that the addition of essential oils cinnamaldehyde and eugenol to organic soil achieved significant reduction of *E. coli* (Yossa et al., 2010a) and *Salmonella* (Yossa et al., 2010b). Further work should be carried out to determine the feasibility of using plant essential oils as a slurry amendment to achieve improved microbiological quality.

1.9. Intentional microbial inoculation

Understanding the factors that affect microbial survival in soil is not only important to reduce transmission of zoonoses, but also to maximise the proliferation of beneficial microbes that are intentionally released to the environment to perform a specific desired function. Such functions include increasing nutrient supply to crops, inhibiting the activity of plant pathogens, improving soil fertility, and bioremediation. Plant growth promoting rhizobacteria (PGPR), such as species of *Pseudomonas*, *Bacillus*, *Rhizobia*, *Enterobacter* and *Aerobacter* carry out many of these desirable functions in the soil rhizosphere. The most well known example is N fixation in leguminous crops (Chanway et al., 1989). Additionally, PGPR can enhance P-solubility, thereby increasing P-availability and crop yield (Rodríguez and Fraga, 1999). PGPR also have a biocontrol function, and offer protection to the crop through production of secondary metabolites which inhibit the activity of plant pathogens. Not only does metabolite

production favour crop growth, it also improves the ecological fitness of the PGPR, thus increasing the likelihood of PGPR establishment post-inoculation. Those PGPR capable of metabolite production avoid top-down control, i.e. predation, and can also compete more effectively with other rhizosphere microorganisms for resources and substrate. Additionally, PGPR benefit from substrate released by protozoa as they graze on other organisms to which they have been deflected (Jousset et al., 2008).

Although these interactions have positive connotations for the agricultural industry, they ultimately provide an insight into the fate of introduced microorganisms, which is highly relevant to eliminating undesirable pathogen invaders from soil.

1.9.1. Carrier materials

Microbial inoculants can also be used for bioremediation purposes. Typically, survival will decline rapidly following inoculation unless the bacteria display ecological selectivity and have some competitive advantage that will favour their proliferation in the soil environment. Microbes that are selected for the purpose of bioremediation will have a unique metabolic capability to utilise the xenobiotic of concern as substrate, thus ensuring that competition will not limit population establishment in the soil. However, in most cases, bacteria will not possess selectivity and will be susceptible to competition from indigenous microflora that will be better adapted to the oligotrophic soil environment. By adding a 'carrier material' to the inoculation mix, some degree of protection is conferred to the microorganism, thus improving chances of survival. The carrier material is selected based on ability to provide a protective, nutrient rich substrate for temporary sustenance of the inoculant. Characteristics of a suitable carrier matrix include a wide distribution, sufficient water holding capacity, consistency of physical and chemical attributes, absence of toxicity and efficient release, thereby ensuring that it is conducive to the survival of the inoculant microorganism and that it is safe for application in the environment (reviewed in Stephens and Rask (2000)).

The use of manure as a carrier may also be effective in promoting the survival of beneficial microorganisms due to its ubiquitous nature and high organic matter content; however, in reality its suitability is compromised by the presence of plant and animal pathogens. Following application of poultry manure inoculated with a suite of PGPM to

a banana crop, a significant increase in shoot and root mass, and N and P content was observed as compared to a control crop without biofertilization. This was attributed to successful root colonisation by *Azospirillum*, *Azotobacter* and P-solubilising bacteria, which subsequently enhanced nutrient availability and uptake by the plant. A linear increase in banana growth was associated with increasing application rate of manure, thereby demonstrating its potential as a carrier in the absence of pathogenic constraints (Rivera-Cruz et al., 2008). Similarly, the use of treated swine manure pellets as a carrier for the biocontrol agent *Trichoderma harzianum* resulted in a reduction in the incidence of damping-off disease in sugar beet induced by *Rhizoctonia solani* following application to non-sterile soils, and consequently enhanced the growth of the crop (Kok et al., 1996).

In light of this potential for manure to act as a carrier for beneficial microorganisms, it is possible that land application may also facilitate prolonged survival of undesirable pathogens inherently present in the organic matrix.

1.9.2. Genetically modified microorganisms

Despite the functions provided by beneficial inoculants, there are also concerns surrounding intentional release of microorganisms, particularly those which have been genetically modified (GMO) to exhibit a desirable trait. Such concerns are related to persistence of GMOs, modification of the indigenous microbial community and consequently, disruption of the ability of these microbes to modulate vital soil processes. For example, it was found that the resistance plasmid RP4 conferred to *E. coli* K12 strain was transferred to indigenous soil bacteria within 24 hours of addition to the soil microcosm (Sørensen et al., 1999). Predation by indigenous protozoa was related to *E. coli* K12 decline; however the conjugative plasmid was observed among indigenous microorganisms throughout the experiment, thus indicating that resident biota may accumulate and store genetic material released to the environment and consequently aid persistence of such material in the microbial community. Also, Austin et al., (1990) noted that the addition of genetically modified *Pseudomonas solanacearum* to a soil microcosm had an impact on the feeding behaviour of higher predatory organisms. A significant decrease in flagellate number was associated with the

presence of a recombinant plasmid in the *Pseudomonad*, which was not observed for non-GMOs. This demonstrates the potential for GMO persistence in soils where suitable predators are lacking. However, work by Recorbet et al., (1992) showed that wild and GMO strains of *E. coli* fared similarly when inoculated individually in sterile and non-sterile soils, but that the GMO strain could not compete effectively when inoculated concomitantly. This suggests that maintenance of engineered attributes is metabolically expensive for the GMO strain, and thus compromises survivability in soil. Such studies highlight the need for site-specific risk assessment to identify the factors that regulate microbial interactions prior to environmental release of genetically engineered microorganisms.

By combining knowledge of factors that affect success of intentional release with that which is already known regarding pathogen survival, it may be possible to anticipate interactions likely to occur between land-spread pathogens, resident biota and prevailing soil environmental conditions.

1.10. Aims, research questions and objectives

Current understanding of pathogen survival in relation to the soil biological context has been extensively reviewed and gaps in the knowledge were identified. It was therefore considered an appropriate research topic. The main aim of this project was thus to identify the relationship between pathogen persistence and soil biotic status. It was hypothesised that there would be an inverse relationship between the diversity and configuration of an extant community and the establishment of exogenous microorganisms introduced into soils. The project aim was deconstructed into a series of research questions intended to collectively test this hypothesis:

1. Does microbial diversity and community configuration limit pathogen establishment in soil?
2. Do land management practices indirectly alter survival rates via induced changes in microbial community structure?

3. How can current guidance pertaining to manure application and harvesting requirements be modified to ensure that the biotic status of the soil is taken into account?

Corresponding objectives were developed in order to answer these research questions:

1. To determine the rate of pathogen decline in soil, and relate this decline to the diversity and complexity of the indigenous microbial community
2. To determine the impact of land management regimes on community structure and consequently on pathogen survival in soil

Chapter 2. General methodology

This chapter details the standard methodology used repeatedly throughout this project. The details of various culture techniques that were adopted to assess survival of model pathogens in soil are described. In addition, the general methods of biomass analysis, namely chloroform fumigation extraction (CFE), and substrate induced respiration (SIR) are also described in this chapter. The PLFA technique used to characterise the phenotypic community fingerprint of experimental soils is discussed. These methods will be referred to within individual experimental chapters where appropriate. Any methodology specific to a particular experiment will be detailed in the materials and methods section of the corresponding chapter.

2.1. Culture work to detect pathogen survival in soil

2.1.1. Background

Culture techniques employ a range of nutritional media to differentiate and selectively grow microorganisms from environmental matrices. These media can be used in liquid broth or semi-solid agar phase, and contain nutrient configurations that favour the growth of specific microbial groups. Selectivity can be enhanced through supplementation with inhibitors such as antibiotics, which are often used to isolate antibiotic-resistant strains. Additionally, the choice of incubation conditions is important, as microbial groups have different time, temperature and atmospheric requirements to achieve optimal growth (Allen et al., 2004). Agar is often used to obtain pure cultures and to demonstrate the presence of specific microorganisms within a sample. Under optimal incubation conditions, the microorganisms of interest will utilise the available nutrients to increase biomass and form visible colonies on the surface of the agar, which can then be quantified (Alef and Nannipieri, 1995).

The use of culture techniques for diversity and community analysis is limited due to the fact that only 1% of microorganisms can be cultured directly from soil (Ritz, 2007). Therefore, they are not deemed appropriate for characterising microbial composition at

a community level. However, they are suitable to enumerate specific microorganisms in soil that are capable of exploiting culture media. These microorganisms can be either indigenous or experimentally added with a view to quantifying a response. Enumeration has been successfully used to quantify pathogen persistence in soil over time in a variety of contexts. The influence of temperature (Semenov et al., 2007), UV irradiation (Hutchison et al., 2004b), soil type (Cools et al., 2001), pH (Himathongkham et al., 1999), and microbiota (Jiang et al., 2002), on pathogen survival in soil and organic wastes has been well demonstrated by extraction and enumeration on specific agars. As pathogen-specific agars were available for the model pathogens used throughout this work, culture techniques were considered appropriate for enumeration. The method described below is adapted from Avery et al., (2005).

2.1.2. Pathogen selection

Four model pathogens were selected to investigate community interactions in this project, namely an environmentally-persistent *E. coli* isolate (Lys 9), *Salmonella* Dublin, *Listeria monocytogenes* and non-tox lux-marked *E. coli* O157. These model organisms were selected as they were considered relevant in terms of public health significance, and they also represented contrasting cellular structures and growth strategies. For example, *E. coli* and *Salmonella* strains were chosen as they are both fast-growing, Gram-negative microorganisms with similar host associations and niche requirements (Winfield and Groisman, 2003). In contrast *L. monocytogenes* is a Gram-positive bacterium, and was included to investigate whether this structural difference would result in different survival profiles. Non-tox lux-marked *E. coli* was chosen as it was initially intended to use luminescence as a measure of cell activity, in addition to plate counts. However, it was not possible to use luminescence in this case due to the masking effect of soil particles, which has been reported previously (Rattray et al., 1990). Despite this, this organism was included in the suite of model pathogen, as it has been shown to be a representative proxy for the toxigenic O157 strain of clinical importance (Bolton et al., 1999), and survival data generated in the context of this work would be useful for comparison with other studies.

2.1.3. Pathogen inoculum preparation

The preparation medium was subject to debate, as to prepare in nutrient-rich media could potentially cause shock to the cells on inoculation to a nutrient-poor soil environment. However, preparation in nutrient-poor media may not produce the cell numbers required for the starting inoculum. As pathogenic microorganisms tend to enter the soil environment in a nutrient-rich slurry matrix, it was decided that the inoculum would be prepared in Luria-Bertani (LB) broth. This broth has been used to prepare *E. coli* cultures for inoculation to environmental matrices previously (Avery et al., 2005; Williams et al., 2007a; Williams et al., 2007b).

Aliquots (100 µl) of an overnight culture of each pathogen were added to 100 ml fresh LB broth and incubated at 37^{o}C for 24 hours on an orbital shaker at 120 rev min^{-1}. This culture was centrifuged and subsequently washed 3 times in sterile ¼ strength Ringers solution, to remove all traces of LB prior to inoculation. The culture was decimally diluted to a factor of 10^{-10} in ¼ strength Ringers solution, and tested for absorbance using a microplate reader. The appropriate dilution was chosen based on the optical density (OD) value that corresponded to approximately 10^{8} CFU. This concentration was assumed sufficient to allow enumeration throughout the experimental period, based on previous work by Avery et al., (2005).

2.1.4. Soil screening

Soils were screened for any natural background signals of the test organisms prior to inoculation. This was done by suspending 5 g of soil in 10 ml sterile ¼ strength Ringers solution, followed by briefly vortexing, shaking for 15 minutes on an end-over-end shaker and finally vortexing a second time. Aliquots of the suspension (100 µl) were then plated onto pathogen-specific agar. Background counts were quantified when present, and these were taken into account in the final calculations.

2.1.5. Pathogen enumeration

Microcosms, consisting of 5 g soil in sterile 40 ml polypropylene tubes, were destructively sampled during the enumeration process. Enumeration consisted of adding 10 ml sterile ¼ strength Ringers to each microcosm and vortexing briefly for 1 minute, followed by 15 minutes on an end-over-end shaker. Microcosms were then vortexed briefly a second time. Serial dilutions were established for each microcosm. This was carried out by adding 100 µl of the soil suspension to 900 µl sterile ¼ strength Ringers solution in sterile microtubes, resulting in 10^{-1} dilution. The 10^{-1} dilution was shaken vigorously prior to further dilution. Selected dilutions from this series were plated onto pathogen-specific agar in triplicate and incubated at the appropriate time and temperature, after which characteristic colonies were counted and recorded (Table 2.1). Survival data was then expressed as CFU g^{-1} soil (dry weight). Counts were conducted on agar plates that contained between 30 and 300 colonies, as per standard techniques (Koch, 1994). The limit of detection was then calculated to be less than $6x10^2$ colonies per gram of soil.

Table 2.1 Culture media and incubation conditions required for enumeration of model pathogens in soil

Model pathogen	Agar (Oxoid, Basingstoke)	Incubation time (hours)	Incubation temperature (^{o}C)	Appearance on media	Reference
S. Dublin NCTC #9676	XLD	18-24	35-37	Red colonies, black centre, H_2S production	Bolton et al., (2011)
L. monocytogenes #1778	Oxford	24-48	35	Brown colonies with aesculin hydrolysis (black zones)	Sagoo et al., (2001)
Non-tox lux marked *E. coli* O157	Sorbitol MacConkey plus 5mg/ml kanamycin	24	35	Colourless colonies (non-sorbitol fermenting)	Williams et al., (2008)
Environmental *E. coli* (Lys 9)	Sorbitol MacConkey	24	37	Pink colonies (sorbitol fermenting)	March and Ratnam (1986)
Non-tox *E. coli* O157 #3704	Sorbitol MacConkey plus Cefixime-Tellurite supplement	24	37	Colourless colonies (non-sorbitol fermenting)	Avery et al., (2005)

2.2. Chloroform fumigation extraction (CFE) to quantify soil microbial biomass

2.2.1. Background

Soil microbial biomass typically accounts for 1-4% of soil OM (Sparling, 1992). It responds rapidly to changing environmental conditions, and is typically linearly correlated with soil organic carbon. Therefore, it is a useful measurement as it provides a real-time indication of soil health (Brookes, 2001). The basis of the technique is to measure the difference in microbial carbon between fumigated and non- fumigated samples. Chloroform is a suitable fumigant as it effectively lyses cells, thus releasing cell contents which can be extracted and analysed. Crucially, chloroform does not apparently alter non-microbial soil OM or influence its decomposability (Jenkinson and Powlson, 1976a).

This technique can be used to analyse a range of sample sizes. It is suitable to quantify different elemental fractions of biomass using appropriate extracts, including N, P, S (Joergensen et al., 2011) and metals K, Na, Mg, Mn, Zn, Cu and Ni (Kahn et al., 2009). Isotopic trace analysis can also be applied in the CFE technique to investigate the flux of various elements through the microbial biomass, e.g. ^{13}C and ^{15}N (Allen et al., 2005). Vance et al., (1987) originally used dichromate digestion to determine microbial carbon liberated by fumigation. This was further developed by Wu et al., (1990), who introduced automated carbon measurement using UV persulphate oxidation, and proposed the inclusion of a conversion factor to account for extraction efficiency when relating microbial carbon to biomass. CFE has been successfully applied in many studies to investigate microbial biomass in response to a variety of parameters, including presence of toxic metals (Brookes and McGrath, 1984), conversion to organic agricultural practice (Berner et al., 2008), and potential temperature increase associated with climate change (Bell et al., 2010).

The method outlined below is based on the original devised by (Vance et al., 1987), according to British standard 7755-4.4.2:1997 (BSI, 1997).

2.2.2. Fumigation procedure

Soil was homogenised and sieved to 4 mm. Soil moisture content was calculated by recording sample weight before and after drying at 105^{o}C for 24 hours. Two aliquots of 10 g were then weighed into glass jars; one of which served as the control or unfumigated sample, and the other as the sample for fumigation. Glass jars containing unfumigated samples were extracted immediately, while those containing samples to be fumigated were uncapped and placed into implosion-resistant desiccators (Corning Pyrex Desiccator ®, UK). Desiccators were prepared by lining with damp tissue paper. A crucible of soda lime was added to the base to absorb any CO_2 present. Finally, a small beaker of ethanol-free chloroform containing anti-bumping granules for smooth boiling was added to the centre of desiccator, which was subsequently sealed by replacing the lid and evacuated using a vacuum pump. Evacuation was continued until the chloroform was visibly boiling within the desiccators for a 2 minute period. The desiccator valves were then closed and the pump was turned off. The desiccators were left in the fume hood for 24 hours to allow effective fumigation and resultant cell lysis to occur. After this time, the desiccators were vented to atmospheric pressure, and the beaker of chloroform was removed from within. The lid was replaced and six consecutive 2-minute evacuations were carried out to ensure that all chloroform was removed from the desiccators.

2.2.3. Extraction procedure

Both fumigated and unfumigated samples were extracted using 4:1 (v/w) of 0.5 M K_2SO_4, and were placed on an orbital shaker for 45 minutes. Samples were subsequently filtered through Whatman No.42 filter papers. The filtrate was then frozen at -20^{o}C until analysis.

2.2.4. Calculation of microbial carbon

Total organic carbon (TOC) concentrations in extracts were determined with a Shimadzu TOC-V_{cph} analyser. This instrument employs a combustion catalytic oxidation method. Briefly, samples were acidified to pH 2 to 3, and bubbled with sparge gas to remove the inorganic carbon (IC) component. The remaining total carbon (TC) was then

measured to determine total organic carbon (TOC), by means of CO_2 detection in a non-dispersive infrared gas analyser. Final results were then expressed as µg CO_2-C g^{-1} soil.

By subtracting the extractable or baseline carbon within non-fumigated samples from the carbon flush obtained following fumigation after a 24-hour period, microbial biomass carbon can be quantified according to the following equation:

$$\text{Soil microbial biomass carbon (SMBC)} = (C_{fum} - C_{non\text{-}fum})/k_{ec}$$

In this equation, C_{fum} is carbon extracted from fumigated soil and $C_{non\text{-}fum}$ is carbon extracted from non-fumigated soil. Subtraction of $C_{non\text{-}fum}$ from C_{fum} gives a measure of microbial carbon (C_{mic}), which can be subsequently divided by a pre-defined conversion factor k_{ec}, to quantify SMBC. This conversion factor is often included to account for extraction efficiency of SMBC (Bailey et al., 2002). The k_{ec} value can vary according to soil type, but 0.45 is typically used for agricultural soils (Wu et al., 1990).

2.3. Substrate induced respiration (SIR) to quantify active soil microbial biomass

2.3.1. Background

Respiration techniques were initially developed to distinguish between fungal and bacterial biomass through selective inhibition (Anderson and Domsch, 1973). Subsequent modification of the original method enabled effective assessment of the biomass carbon content within the active microbial population (Anderson and Domsch, 1973). The SIR method uses a readily available carbon source to stimulate microbial activity, resulting in enhanced respiration. It operates on the principle that the initial rate of CO_2 production following substrate addition is directly proportional to the microbial biomass within the sample. The method requires the sample to be completely saturated with substrate and the response must be quantified before any increase in microbial biomass can occur (Bailey, 2007).

The SIR technique has been demonstrated as an effective technique to quantify active biomass across a range of treatments and experimental conditions. For example, Ross et al., (1995) demonstrated the effect of seasonality on biomass, and a number of studies have employed this technique to examine biomass response to various land management regimes (Brake et al., 1999; Graham and Haynes, 2005; Govaerts et al., 2007).

The method used here is based on that originally described by Anderson and Domsch (1978), with modifications according to West and Sparling (1986) and International Standard 14240-1:1997 (ISO, 1997).

2.3.2. Sample preparation

Soils were stored at 4^{o}C until use, at which time they were homogenised and sieved to 4 mm. Soil moisture content was calculated by recording sample weight before and after drying at 105^{o}C for 24 hours. Soils were then pre-incubated overnight at 20^{o}C to allow for microbial acclimatisation and recovery of dormant cells. Samples were prepared by placing aliquots of 10 g soil (wet weight) into 150 ml sterile glass hypovials. The samples were sealed using aluminium crimp tops with rubber septa, and were pressurised by adding 30 ml CO_2 free air. This was done to account for the loss of pressure caused by taking headspace samples during incubation. Samples were subsequently placed into an incubator at 20^{o}C for 26 hours. The first headspace sampling was carried out after 24 hours (T_0) to calculate basal respiration rate, which served as a baseline measurement.

The volume of liquid required to adjust the soil to 60% water holding capacity (WHC) was calculated and a glucose solution was prepared to deliver 5 mg glucose g^{-1} soil within this volume. This glucose concentration was assumed sufficient to saturate the soil with carbon, based on previous findings (Anderson and Domsch, 1978; West and Sparling, 1986; Lin and Brookes, 1999). This provides an excess of glucose to ensure that there is no carbon limitation during the test period. The solution was sterilised by autoclaving at 121^{o}C for 15 minutes, and added to the soil after the first headspace sampling at T_0 using a sterile syringe and needle. This soil was mixed by gentle shaking

to ensure even distribution of the solution throughout the sample. The hypovials were then incubated for a further 4 hours to determine SIR rate.

2.3.3. Headspace sampling

Headspace samples were taken three times during this incubation period. The first sample was taken after 24 hours incubation (T_0) to determine the rate of non-induced respiration, i.e. basal respiration rate. This measurement served as a baseline control. The subsequent samplings were carried out following glucose addition, initially after 2 hours incubation (T_2) and again after 4 hours incubation (T_4). This allowed the rate of CO_2 evolution in response to the substrate to be measured, i.e. SIR rate.

To take a headspace sample, the hypovials were removed from the incubator and placed onto the bench. A needle attached to a 20 ml syringe was pushed through the rubber septum and the syringe was flushed with headspace gas a couple of times before taking a 10 ml sample. This sample was ejected into a previously evacuated 7 ml glass GC vial with screw cap and rubber septum. The GC vial was over pressurised to ensure that any potential leak would not result in immediate loss of sample. The headspace samples were clearly labelled according sample ID and the time at which they were taken, and were stored at room temperature until they could be analysed by gas chromatography.

2.3.4. Gas chromatography procedure

The instrument used for CO_2 analysis was the Varian CP-3800 Gas Chromatograph (California, USA), with a CTC Analytics Combi-pal auto sampler. The instrument was calibrated using certified Argo CO_2 gas in a range from 500-1000 ppm. The sample was injected into a column injector at 60^{o}C and then carried through the column, packed with Porapak™ packing (80-100 mesh) by high purity helium carrier gas (He BIP, Air Products, Dublin, Ireland).

2.3.5. Calculation of microbial carbon

The rate of CO_2 evolution was calculated at T_0, T_2 and T_4 according to the following equation:

$$RCO_2\text{-}C\ (\mu g\ CO_2\text{–}C\ g^{-1}\ h^{-1}) = (V_0 \times 12 / (22.4 \times t \times m_{sd}))*1000$$

where RCO_2-C represents rate of CO_2-C formation on a soil dry mass basis (µg CO_2-C g^{-1} h^{-1}), V_0 is enrichment volume of CO_2 under standard temperature and pressure conditions (ml), 12 is molar mass of CO_2-C (g mol^{-1}), 22.4 is molar volume of CO_2 under standard conditions, t is incubation time (hours) and m_{sd} is mass of dry soil (g). The average background CO_2 concentration was subtracted to take atmospheric input into account. The final induced rate was calculated by subtracting the RCO_2-C value obtained at T_2 from that obtained at T_4.

2.4. Phospholipid fatty acid (PLFA) analysis to determine soil microbial community structure

2.4.1. Background

All living cells contain PLFAs within their membranes, which degrade to diglycerides on cell death (White et al., 1979). Therefore, these fatty acids solely represent the living fraction of the microbial biomass, and can be effectively used as a tool in environmental analysis (Salomonová et al., 2003). Phospholipids are comprised of glycerol molecules, which are attached to fatty acid chains and phosphate groups at either end, thus creating hydrophobic and hydrophilic regions, respectively, and conferring a characteristic asymmetric shape to the lipid. The variation in the number of carbon atoms and double bonds on the hydrophobic tail are used to differentiate between phospholipids, giving rise to a detailed nomenclature (Kaur, 2005).

The type and proportion of phospholipids differ between microbial groups, thus providing a ‘fingerprint’ of the microbial community (Bossio et al., 1998). For example,

Gram-positive bacteria are characterised by branched chain fatty acids, whereas Gram-negative bacteria are typically identified using cyclopropane fatty acids. Linoleic acid has been shown to account exclusively for the fungal proportion of the community (Zelles, 1999). Therefore, PLFA is often used to characterise phenotypic community structure in soil. The phenotype is an important descriptor of diversity as it is the environmental expression of the genotype; thus it accounts for microbial interactions with the environment, and reflects the physical manifestation of the community. The use of PLFA overcomes limitations associated with non-cultivable organisms as it can detect a greater proportion of the total soil community.

The PLFA method has been shown to be sensitive to community shifts associated with various environmental treatments, including the effect of organic amendments (Frostegård et al., 1997; Esperschütz et al., 2007), long-term agricultural management (Jangid et al., 2008), rhizosphere associations (Steer and Harris, 2000; Patra et al., 2008), season and soil type (Bossio et al., 1998) and contamination (Frostegård et al., 1993). The PLFA method was considered appropriate for this work as species-level information was not required, but rather a means characterising the actual community context which introduced pathogens encounter.

The PLFA method used throughout this project was based on the method originally described by Bligh & Dyer (1959), and adapted according to modifications by White et al., (1979) and Frostegård (1991). Freeze-dried soil was used throughout this analysis. All chemicals originated from and all solvents used were Hypersolv^{TM} grade unless stated otherwise.

2.4.2. Extraction

Soil for PLFA analysis was collected and stored at 4^{o}C until required. It was sieved to 4 mm and frozen at -80^{o} C overnight, before freeze drying on a Scanvac Coolsafe 55-4 at -57^{o}C. The freeze-dried soil was then stored at -20^{o} C until such time as PLFA could be carried out.

Freeze dried soil was weighed out in 10 g aliquots and placed into glass media bottles. Citrate buffer solution was prepared by combining 0.15 M of citric acid dihydrate (31.5 g l^{-1}) and 0.15 M of trisodium citrate (44.1 g l^{-1}) in deionised water. This solution was adjusted to pH 4 by the addition of dilute acetic acid. Bligh and Dyer solution was then prepared by combining citrate buffer, chloroform and methanol in the ratio of 0.8:1:2 (v/v/v), respectively. Approximately 50 mg l^{-1} of butylated hydroxy toluene (BHT/ 2, 6, di-tert butyl-4-methylphenol) was added to the final Bligh and Dyer solution as an anti-oxidant. Bligh and Dyer solution was then added to the freeze-dried soil. The amount of Bligh and Dyer added to each sample varied between 15 ml and 20 ml, depending on a visual estimate of OM content within the sample. Samples that appeared to have relatively higher OM received a larger volume of Bligh and Dyer solution. The sample vial was then covered with PTFE tape and the cap was screwed on tightly over the tape, to create a barrier between the sample and the plastic cap of the vial, thus preventing contamination by plasticiser. Sound energy was used to disrupt bacterial cells within the soil by sonicating samples for 30 minutes, followed by shaking for 30 minutes on an end-over-end shaker. Samples were then centrifuged at 2000 rpm for 10 minutes, and the supernatant was poured into a brand-new clean glass vial. A further 4 ml of citrate buffer and 4 ml of chloroform was added to the supernatant, resulting in a separation of the liquid into two phases. The sample was then refrigerated overnight to allow a clear interface to establish between the phases. The aqueous upper layer was then removed and discarded via Pasteur pipette, and the remaining organic layer containing the lipid fraction was dried down on a Techne DB3 sample concentrator with Dri-Block^{TM} heater set at 37^{o}C, under a constant stream of N. The dried sample was stored at -20^{o}C until such a time as fractionation could be carried out.

2.4.3. Fractionation

The fractionation procedure was carried out to separate neutral, glycolic and polar lipids. This was done by solid phase extraction (SPE) using 3 ml / 500 mg silica Sep-pak Vac^{TM} columns (Waters chromatography, Santry, Dublin). Approximately 0.5 g sodium sulphate was added to the top of each SPE column, and the columns were then placed on to the SPE manifold, which was attached to a vacuum pump. The columns

were conditioned by washing through with 2 ml of methanol, acetone and then chloroform under vacuum, which was collected in waste vials within the manifold. The vacuum pump was then left on for a few minutes to ensure that any residual solvent was removed from the column, the vacuum pump was switched off and a final 2 ml of chloroform was allowed to seep through under the force of gravity.

At this point, the frozen samples were defrosted at room temperature and were reconstituted with 1 ml of chloroform by washing down each side of the sample vials. Reconstituted samples were then loaded onto SPE columns, and the samples were subsequently washed through the columns with solvents of increasing polarity to separate the lipids. Firstly, 5 ml of chloroform was added to the top of the columns to elute the neutral lipids. This was followed by elution of the glycolic lipids with 12 ml of acetone. The vials in the manifold were then removed and the contents were discarded as waste. Finally, clean labelled sample vials were placed in the manifold to collect the polar lipids containing the phospholipids of interest, which were eluted with 8 ml of methanol.

The methanol was then evaporated, and the phospholipid fraction was dried down on a sample concentrator set at 37^{o}C under a constant stream of N. The samples were then stored under N at -20^{o}C until such a time as mild alkaline methanolysis could be carried out.

2.4.4. Mild alkaline methanolysis

The purpose of mild alkaline methanolysis procedure was to separate the fatty acid from the phosphate backbone of the phospholipid, and replace the glycerol bonds with a methyl group. This results in the formation of fatty acid methyl esters which are suitable for analysis using gas chromatography.

The presence of water could potentially interfere with the derivatisation process by displacing methanol and bonding with the fatty acids. This would result in the formation

of free fatty acids rather than the desired methyl esters that are suitable for analysis. Therefore, solvents were dried under anhydrous sodium sulphate prior to use.

The frozen polar lipid samples were defrosted at room temperature and reconstituted with 1 ml of a 1:1 (v/v) mix of toluene (Analar):methanol. Reconstituted lipids were subsequently hydrolysed by adding 1 ml of 0.2M methanolic potassium hydroxide. The samples were mixed by gentle swirling action and were incubated at 37^{o}C for 30 minutes. Following incubation, 0.25 ml of 1 M acetic acid was added to the samples. This raised sample pH to neutral, and stopped the hydrolysis reaction. A 4:1 (v/v) mix of hexane: chloroform was prepared and 5 ml of this solution was added to samples, followed by addition of 3 ml of deionised water. Samples were then sonicated for 30 minutes and refrigerated overnight to facilitate clear separation into two phases.

2.4.5. Clean-up procedure

The aqueous lower layer was removed from each sample using Pasteur pipette. A 0.3 M sodium hydroxide solution was prepared in deionised water (12 g l^{-1}), and 3 ml of this solution was added to the samples to increase pH. This caused the polarity of any remaining un-derivatised fatty acids to increase, thus reducing solubility in the organic phase. The top layer of the sample was filtered by Pasteur pipette through anhydrous sodium sulphate using Whatman No.4 filter papers, and the filtrate was collected in a brand-new clean glass vial below. The sample was dried down on a sample concentrator set at approximately 25^{o}C under a constant stream of N. The dried sample was stored at -20^{o}C until such a time as final sample preparation for GC analysis could be carried out.

2.4.6. Sample preparation

Frozen samples were defrosted at room temperature and reconstituted with 200 µl hexane, by washing down both sides of the glass vial. The samples were subsequently transferred to amber GC vials containing 100 µl spring inserts. The vials were then crimped with aluminium seals, and labelled accordingly. The samples were stored at -20^{o}C until such a time as they could be analysed on the GC.

2.4.7. GC procedure

The GC which was used to separate the fatty acid methyl esters was an Agilent Technologies 6890N, fitted with Varian Star software. A HP-5 capillary column was used with a split/splitless injector. The column specifications were as follows: 30 m length, 0.32 mm ID, and 0.25 µm film consisting of 5% phenylmethyl siloxane. The sample was passed through the column via helium carrier gas at a flow rate of 1 ml min^{-1}. An autosampler was used to aspirate 1 µl of sample and inject into the GC at an injector temperature of 310^{o}C. The temperature programme used to separate the FAMEs commenced with a splitless hold time of 1 minute at 50^{o}C. The temperature then increased to 160^{o}C at a rate of 25^{o}C min^{-1}. It then further increased to 240^{o}C at a rate of 2^{o}C min^{-1}, followed by a final increase to 310^{o}C at a rate of 25^{o}C min^{-1}. A flame ionising detector (FID) was used to detect the FAMEs at an operating temperature of 320^{o}C.

2.4.8. Peak identification

Varian Star software was used to analyse the separated fatty acids. Retention times for fatty acids within samples were as compared to retention times of a standard mixture containing 26 known fatty acids (SUPELCO, Sigma-Aldrich, UK). Raw data was then standardised by expressing individual peak areas as a percentage of the total peak area of the sample chromatogram, which gave results on a mol percent (mol %) basis. Results in this format were then suitable for further statistical analysis.

2.5. Statistical analysis

2.5.1. One-way analysis of variance (ANOVA)

One-way ANOVA was used to investigate statistically significant differences in biomass using CFE (Chapter 4 and 6) and SIR (Chapter 4) data. Statistica software (Version 10) was used to carry out this analysis.

2.5.2. Principal components analysis (PCA)

Principal components analysis (PCA) was used to analyse mol % data from PLFA profiles in Chapters 4, 5 and 6. As variability between replicates was generally quite high, the correlation rather than covariance method of analysis was used in PCA. The correlation method serves to standardise the data. The basis of the PCA technique is to reduce the dimensionality of multi-variate datasets while retaining characteristic variability between such datasets. It effectively condenses the dataset into principal components (PC) that explain the entirety of variation that exists within the dataset. This technique has been applied to PLFA data in a number of studies, e.g. Bossio et al., (1998); Esperschütz et al., (2007); Lejon et al., (2007). ANOVA was subsequently carried out on the PC values to determine significant differences in community structure between treatments. Statistica software (Version 10) was used to carry out this analysis.

2.5.3. Non-linear estimation

Mean CFU values were used to calculate the death rate (k-value) for all model pathogens in Chapters 4 and 6 (Statistica, Version 10). A first order decay function was applied to the data according to the equation $Y=A+B*\exp^{-kt}$, where Y represents the population (CFU g^{-1} soil dry weight) at a given time t, A+B denotes the apparent starting concentration of cells (i.e. intercept with the y-axis), A denotes the asymptote of the final population concentration, and k denotes the death rate (d^{-1}). This function has been used previously to estimate pathogen decay rates (Mubiru et al., 2000; Oliver et al., 2006).

Chapter 3. The impact of soil type, biology and temperature on the survival of *E. coli* O157

3.1. Introduction

Escherichia coli are Gram-negative bacteria that belong to the family Enterobacteriaceae (Winfield and Groisman, 2003). Humans are colonised by commensal *E. coli* at birth, and these harmless strains of the bacteria subsequently comprise part of the natural intestinal microflora (Jones, 1999). However pathogenic strains also exist, including enterohaemorrhagic *E. coli* (EHEC), which can cause severe illness in humans. These *E. coli* form part of the verocytotoxigenic (VTEC) group, so-called due to the production of cytotoxins that inhibit protein synthesis within vero cells of eukaryotic organisms. Alternatively, this group can be termed Shigatoxigenic (STEC), as they produce Shiga-toxins similar to those produced by *Shigella dysenteriae* (EFSA, 2007). Infection with EHEC can be asymptomatic, but can also induce non-bloody and bloody diarrhoea, hemorrhagic colitis, haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic pupura (Su and Brandt, 1995). The majority of human illness is caused by the O157 serotype, though cases of non-O157 VTEC infection are becoming more common (EFSA, 2007). For example, a large outbreak occurred in Germany in 2011 which was caused by Shiga-toxin producing *E. coli* O104:H4. This non-O157 strain caused over 3000 cases of diarrhoeal disease and 773 cases of HUS, which resulted in up to 50 deaths between May and July (EFSA, 2011).

Ruminant animals, particularly cattle, constitute the primary reservoir of VTEC among livestock. Chapman et al., (1997) found that that the prevalence of *E. coli* O157 in cattle, sheep, pigs and poultry was 15.7%, 2.2% 0.4% and 0% of animals, respectively, demonstrating the importance of cattle in VTEC infection. Shedding is variable and dependent on factors such as season, age, stress, feeding regimen and animal husbandry (Rugbjerg et al., 2003; Chase-Topping et al., 2007). The highest shedding rate among cattle is observed in the warmer months of summer and early autumn, which coincides with the onset of human infection (Ogden et al., 2004). Young livestock typically shed greater concentrations of *E. coli* O157 than adult animals (Cray and Moon, 1995). In this study, calves and adults were inoculated with 10^{10} CFU, and

faecal concentrations were investigated 3 days post-inoculation. Shedding by calves ranged from 10^6-10^7, as compared to a range of 10^2-10^7 for adults. On average, calves shed more *E. coli* O157 than adults by an order of magnitude. Calves also shed for a longer duration. The difference between adults and calves was partly attributed to age-related rumen conditions and functionality in this case (Cray and Moon, 1995).

Dietary stress, and stress associated with weaning and movement have been identified as risk factors involved in shedding (Chase-Topping et al., 2007). Additionally, the type of feed used can influence the concentration of *E. coli* O157 shed by livestock. It has been suggested that a grain-based diet tends to promote acidic conditions within the gut of the animal, which favours proliferation of the O157 strain, and thus increases the likelihood of shedding (Diez-Gonzalez et al., 1998). Switching the diet to forage has been recommended to reduce incidence of *E. coli* O157 at slaughter; however the benefits of this method have been subject to much debate (Hovde et al., 1999; Russell et al., 2000).

Following release, either by direct defecation or land-spreading, VTEC may be transmitted from/between animals to/and humans via the faecal-oral route. The infective dose of *E. coli* O157 is very low and as few as 10 cells are required to cause illness (Willshaw et al., 1994). Infectious dose is highly contingent on host susceptibility, which is influenced by a number of factors including immunosuppression, aging and malnutrition (Morris and Potter, 1997). It has also been shown that the infectious dose is contingent on the ability of the organism to withstand acidic conditions in the human gut (Benjamin and Datta, 1995).

E. coli may be transmitted directly through contact with farm animals and accidental ingestion of contaminated soil and slurry particles. For example, 4 cases of bloody diarrhoea and HUS occurred throughout Nottingham and Leicester in 1994. The common thread between these individuals was a trip to a farm visitor centre where direct contact with infected animals took place. Transmission was attributed to lack of adequate hand-washing facilities and hygiene information in this case (Shukla et al., 1995). In 2002, a child became infected with toxigenic *E. coli* O157 following a trip to a petting zoo. Further investigation found that other petting zoos also tested positive for this organism, highlighting the potential role of these amenities in *E. coli* transmission (Heuvelink et al., 2002a). An outbreak of *E. coli* occurred in the UK in 1997 following

the Glastonbury music festival. This was associated with camping on land previously grazed by cattle, and contact with contaminated mud was identified as the source of infection (Crampin et al., 1999).

Alternatively, indirect transmission may occur through ingestion of contaminated environmental media such as crops and water supplies. An outbreak of HUS associated with Shiga-toxin producing *E. coli* occurred in Sweden in 2008. This was sourced back to a crop of lettuce. The crop had been irrigated with contaminated water, which had been collected downstream from a cattle-grazed farm. The cattle tested positive for pathogenic *E. coli*, which was identical to the outbreak strain (Söderström et al., 2008). Islam et al., (2005) showed that *E. coli* O157 could survive in carrot fields for up to 196 days following application of contaminated poultry manure and dairy cattle manure, demonstrating the potential of crops as a vector in pathogen transmission. Thus, as an interface between faecal deposition and human exposure, the soil matrix plays a key role in regulating pathogen populations.

Research on pathogen survival in soil has generally focused on abiotic factors that promote inactivation. Persistence is typically associated with cool, moist conditions (Jenkins et al., 1999; Cools et al., 2001; Habteselassie et al., 2008) where desiccation and exposure to UV irradiation is limited (Hutchison et al., 2004b). Fine textured soils with a well developed microstructure tend to confer protection to introduced microorganisms, thus prolonging survival (Rutherford and Juma, 1992; Wright et al., 1995). Extremes of pH promote inactivation; whereas survival is favoured by strong CEC (Coleman et al., 2004) and high OM content (Habteselassie et al., 2008).

In addition, various biotic factors involving predation, competition and occupation of functional and physical niche space also act on the pathogen within the soil matrix, thus partially determining its fate. There has been limited research into the effect of the soil microbial community on pathogenic *E. coli* survival. For example, Jiang et al., (2002) showed that *E. coli* O157 fared better in manure-amended autoclaved soil as compared to manure-amended unautoclaved soil. This observation was attributed to antagonistic interactions between *E. coli* and resident microbiota. Similarly, van Elsas et al., (2007) used differential fumigation to manipulate microbial diversity within soil samples, and subsequently inoculated the soil with non-toxigenic *E. coli* O157. This work showed an inverse relationship between diversity and pathogen

survival. However, further research is necessary to disentangle the complex biotic interactions that control *E. coli* O157 within soil, with a view to minimising this pathogen in the environment.

The objective of this experiment was to determine the effect of soil biology and soil type on the survival of a non-toxigenic strain of *E. coli* O157. Fate of *E. coli* was investigated in relation to a shift from cold to ambient temperatures. This was carried out to approximate springtime conditions when livestock manures are applied. It was hypothesised that:

1) *E. coli* concentration would remain stable at low temperatures, irrespective of native community status, due to limited microbial activity
2) An increase in temperature would cause an increase in the *E. coli* concentration in sterile soil due to the absence of competition from a native microbial community
3) An increase in temperature would cause a decrease in the *E. coli* concentration in non-sterile soil due to enhanced activity of the native microbial community and associated competition and antagonism
4) *E. coli* would survive better in clay as compared to sandy loam soil due to more favourable conditions associated with microhabitat and nutrient availability.

3.2. Materials and methods

3.2.1. Soil collection and preparation

Two contrasting soil types from the Bearsted and Evesham series were used for this experiment. These soils are both classed as Cambisols according to World Reference Base (FAO, 2006), respectively. The Bearsted is a typical brown earth, described as coarse loamy passing to sandstone. It has a pH of 6.07, total N of 1.25 g kg^{-1}, total C and N (%) of 1.0 and 0.1, respectively, and a C/N ratio of 10.6, based on previous analysis (Jeffery et al., 2007). The Evesham is a typical calcareous pelosol, described as clayey passing to clay or soft mudstone. It has a pH of 6.6, total N of 2.58 g kg^{-1}, total C and N (%) of 2.9 and 0.2, respectively, and a C/N ratio of 13.9, again based on previous analysis (Jeffery et al., 2007). Soils were collected from Silsoe Experimental Farm, Cranfield, UK. Bearsted soil was collected from Showground Field (X507500,

Y235500), and Evesham soil was collected from Ivy Ground Field (X507700, Y235200). Samples were taken from the top 15 cm of a grass field margin, which had not been grazed or received livestock manure application within the last 10 years. Soil was sieved to 4 mm and well mixed. The WHC for each soil was determined by the method described by Franz et al., (2011). Moisture content was then adjusted so that soils exhibited similar cohesiveness to achieve standard friability between different soil types, by wetting-up or drying on the bench as appropriate. Following adjustment, soil moisture was measured by oven-drying at 105^{o}C for 24 hours, and expressed as a percentage of WHC. This was determined to be 25% and 44% of WHC for the sandy loam and clay soils, respectively.

Sub-samples (5 g) were transferred to microcosms consisting of sterile polypropylene vials (40 ml). Half of these microcosms were sterilised by autoclaving twice over a 2 day period (121^{o}C for 1 hour at 1 bar). The remaining microcosms were not sterilised and had an intact microbial community. Microcosms were incubated at 4^{o}C for the first 6 days of the experimental period, and at 18^{o}C thereafter. This temperature regime was chosen to simulate livestock manure application during a cold period followed by a period of warming, which would be typical of spring conditions in Ireland.

3.2.2. Pathogen inoculation and survival analysis

The test organism used for this work was *E. coli* O157:H7 strain #3704, obtained from Dr. Lisa Avery at The Macaulay Land Use Research Institute, UK. It was shown to be non-toxigenic by Verocell assay and PCR. It is an environmental strain which was originally isolated from a sample taken from a farm drain (Campbell et al., 2001).

A preliminary inoculum culture was propagated and serially diluted to find out which dilution would contain approximately 10^{8} cells. This inoculum was prepared by adding 100 µl of an overnight culture of *E. coli* O157 to 100 ml fresh Luria-Bertani broth and incubating at 37^{o}C for 24 hours on an orbital shaker at 150 rev min^{-1}. This culture was subsequently centrifuged at 10 000 x g and washed three times in sterile ¼ strength Ringers solution. The culture was then serially diluted to a factor of ten, and each dilution was plated onto Sorbitol MacConkey agar amended with Cefixime-

Tellurite supplement (CT-SMAC), which is selective for the O157 strain. Plates were incubated at 37^{o}C for 24 hours. These plate counts were used to identify the dilution containing the appropriate cell concentration for microcosm inoculation.

A final inoculum culture was then prepared, as described above, and 100 µl of the appropriate dilution, as identified previously, was added to test microcosms. This inoculum delivered a total of 10^8 cells, which corresponded to 10^7 cells g^{-1} soil, and resulted in a final moisture content of 30% and 47% of WHC in sandy loam and clay soils, respectively. This dilution series was plated subsequent to inoculation, to confirm cell concentration within the final inoculum and to account for any potential variation in cell growth between preliminary and final inocula. This variation was accounted for in the calculations (adapted from Avery et al., (2005)).

Control microcosms were inoculated with 100 µl of sterile ¼ strength Ringers solution. All microcosms were mixed gently by hand following inoculation. They were sampled destructively immediately after inoculation (denoted T_0), and on days 2, 4, 8, 16, 32 and 64 of the experimental period (denoted T_2, T_4, T_8, T_{16}, T_{32} and T_{64}). They were monitored for evaporation by weighing, and sterile water was added when necessary. This experimental set-up consisted of two soil types, two treatments (sterile and non-sterile), both with and without *E. coli* addition in triplicate, amounting to 24 microcosms for each sampling date, and 168 microcosms in total. Enumeration was carried out as described in Section 2.1.4.

3.2.3. Statistical analysis

E. coli counts were reported as CFU g^{-1} soil (dry weight). Average CFU values were calculated based on triplicate samples and used in statistical analysis (SAS, Version 9.1). This experiment investigated interactions between temperature, treatment and time, and was therefore conducted in a 2x2x7 factorial design, with soil as a blocking factor. The Mixed Procedure in SAS was used to determine the effect of soil type, treatment and time interactions on *E. coli* O157 survival. All assumptions of the analyses were met. Values obtained at 4^{o}C and at 18^{o}C were analysed separately, to investigate *E. coli* response to warming temperatures that may be encountered during spring land applications. Soil types were also analysed separately to determine the effect

of treatment over time. Finally, this procedure was used to determine the effect of the interaction between treatment and incubation temperature on survival.

Mean CFU values were also used in Statistica (Version10) to calculate the death rate (k-value) for *E. coli* in non-sterile sand and clay during incubation at 18^{o}C. The technique and decay function used in this analysis are both described in Chapter 2.5.3.

3.3. Results

The survival characteristics of a non-toxigenic strain of *E. coli* O157 added to sandy loam and clay soil types with contrasting biological composition were investigated (Fig. 3.1). *E. coli* concentrations were stable at 4^{o}C and there was no significant effect ($P>0.05$) of time, treatment, soil type, or their interaction, on survival. Conversely, the three-way interaction between time, treatment and soil type was strongly significant at 18^{o}C ($P<0.05$). This interaction showed that in sterile soil where biology was absent, *E. coli* concentrations increased, whereas in non-sterile soil where biology was present, *E. coli* concentrations decreased.

Samples were moved from 4^{o}C to 18^{o}C on Day 6 of the experimental period, and the first sampling at 18^{o}C took place at T_8. There was a lag period before the interaction became apparent, in both sandy loam and clay, as there was no significant difference between sterile and non-sterile soils at T_8 for either soil type. In sterile soil, *E. coli* populations increased in both soil types during incubation at 18^{o}C. This increase was significant initially; however, populations stabilised toward the end of the experiment. There was no significant difference in *E. coli* concentration in clay between T_{16} and T_{64} at 18^{o}C. Similarly, there was no significant difference in *E. coli* concentration in sandy loam between T_{16} and T_{32} at 18^{o}C; however, *E. coli* increased significantly thereafter until the final sampling date at T_{64}. Overall, there was a significant increase in *E. coli* concentrations in both soil types between T_8 and T_{64} at 18^{o}C ($P<0.05$).

In non-sterile soil, *E. coli* populations decreased in both soil types during incubation at 18^{o}C. This decrease was maintained throughout, and was significant between each sampling interval for both soil types ($P<0.05$).

There was also a significant effect of the treatment by temperature interaction on *E. coli* survival ($P<0.05$), but there was no difference in this effect between soil types. The interaction was significant for all combinations of treatments and temperatures, with the exception of non-sterile and sterile soils during incubation at 4°C. Similarly, there was no significant effect of this interaction between sterile soils incubated at 4°C and 18°C.

Death rates were calculated for *E. coli* in non-sterile sand and clay during incubation at 18°C. The k-value obtained for clay was 0.36 d^{-1}, as compared to a k-value of 0.23 d^{-1} for sandy loam.

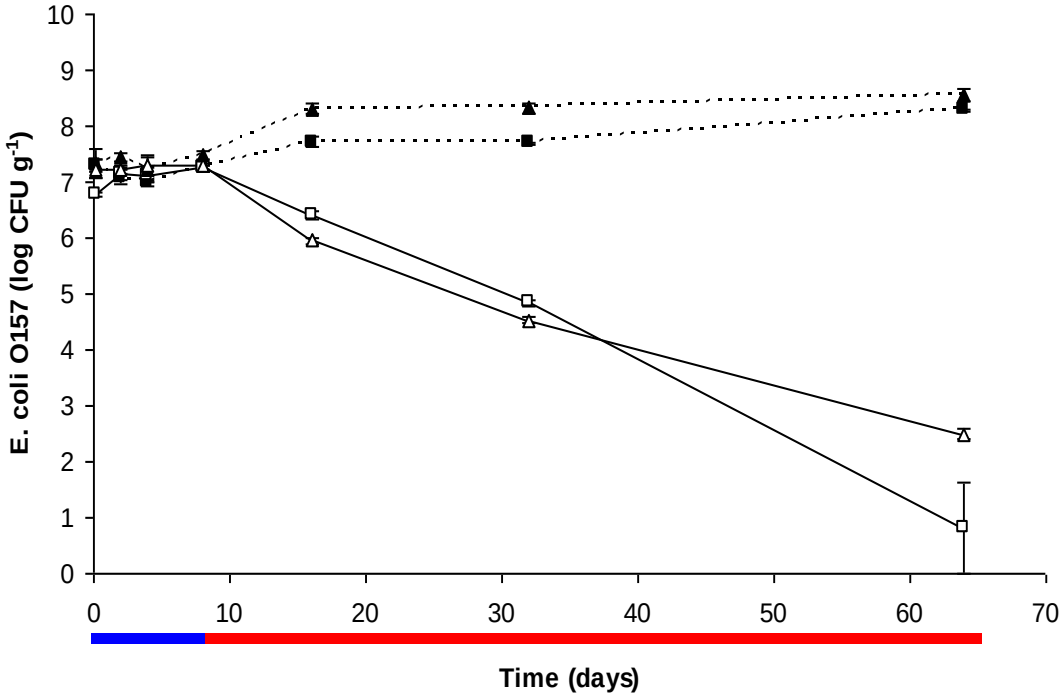


Fig. 3.1 Survival of *E. coli* O157 in sterile and non-sterile sand and clay microcosms. Values represent log_{10} transformed means of plate counts (CFU g^{-1} soil, dry weight), ± standard error (n=3). Note that error bars fall within confines of treatment symbols in some instances. Dashed ▲=sterile clay, dashed ■=sterile sand, solid Δ=non-sterile clay, solid □=non-sterile sand. Incubation at 4°C is represented by the blue temperature bar; incubation at 18°C is represented by the red temperature bar

3.4. Discussion

E. coli O157 is a pathogen of public health concern that is ubiquitous in the agricultural environment. Therefore, it is important to identify the factors that influence its survival with a view to minimising associated risks. The objective of this experiment was to investigate the impact of soil biology, temperature and soil type on the survival of a non-toxigenic strain of *E. coli* O157. It was hypothesised that *E. coli* response would be contingent on the soil biological context. It was also hypothesised that this response would change according to temperature conditions, and would differ between soil types. It was found that *E. coli* grew in sterile soil in the absence of a microbial community, and that *E. coli* declined in non-sterile soil where the soil community remained intact. This treatment effect was not apparent at 4^{o}C and only became significant with a temperature shift to 18^{o}C. This temperature regime was chosen to reflect warming conditions which tend to occur in Irish soils following livestock manure application in late spring. It was also shown that there was an association between soil type and *E. coli* survival, but as soil was used as a blocking factor in this experimental design, it was not possible to delineate any significant effects.

E. coli concentrations were stable at low temperature, irrespective of soil biotic status. A notable change was observed from T_8 onwards, when samples were incubated at 18°C. This resulted in a decrease in *E. coli* O157 concentration in non-sterile systems and a corresponding increase in sterile systems. The observed decrease was likely due to enhanced activity of indigenous soil microorganisms at 18^{o}C, and resultant antagonism toward the introduced pathogen. The effect of temperature on interactions between pathogens and the soil community has been reported previously. Jiang et al., (2002) observed a more rapid decline of *E. coli* O157 in manure-amended unautoclaved soil at 21^{o}C as compared to 5^{o}C. This was attributed to an increase in microbial activity with temperature and consequently, greater competition for nutrients. Similarly, Vidovic et al., (2007) found that *E. coli* O157 declined more rapidly at 22^{o}C as compared to 4^{o}C in unautoclaved soil. In this case, it is likely that the introduced *E. coli* population was at a disadvantage as it was not adapted to the oligotrophic soil environment. Thus, it was less capable of using available substrate and less competitive than the indigenous soil

community. This competitive interaction was minimal at 4^{o}C due to limited metabolic activity, but became significant at 18^{o}C.

Predation of introduced *E. coli* by the soil community may have also played a role in the observed decline in non-sterile soil at 18^{o}C. Sørensen et al., (1999) found that an introduced strain of *E. coli* K12 persisted in soil for 70 days at 4^{o}C and 10^{o}C; however, it was no longer detectable after 20 days at 25^{o}C. The decline in *E. coli* K12 occurred with a corresponding increase in indigenous soil flagellates and ciliates. Similarly, Recorbet et al., (1992) noted that a decrease in the concentration of introduced *E. coli* coincided with an increase in the indigenous amoeba population.

These results show that biotic suppression of livestock manure pathogens will only occur as temperatures increase if a fully intact and functioning microbial community is present.

An increase in *E. coli* concentration was observed in sterile clay and sandy loam soils in the absence of competitive and predatory interactions. The sterilisation process can alter the physical and chemical environment of the soil. Razavi and Lakzian (2007) found a significant increase in the concentration of extractable organic carbon in autoclaved soil as compared to a control using the CFE technique. This increase was attributed to the breakdown of humic substances and the death of microorganisms. Also, Unc and Goss (2006) observed a significant increase in the number of *E. coli* in manure-amended sterile soil. This was due not only to the elimination of competitors and predators during sterilisation, but also to the extra carbon released by cell lysis. In this case, the additional carbon may have served as an important food source for the pathogen.

The upward trend in *E. coli* concentration in both sterile soil types was maintained until the end of the experiment. If the experiment had continued over a longer time period, it is possible that the concentration would have reached equilibrium, resulting in a plateau and eventual decline. This is because soils have an inherent carrying capacity, related to the availability of nutrients and habitat space within the matrix. Once the carrying capacity is reached, population increase can no longer be sustained (Recorbet et al., 1992).

Soil type is another important aspect of pathogen survival, as different soils have unique biogeochemical, structural and textural properties that determine the availability of substrate, water and habitats. As soil type was used as a blocking factor in this experimental design, it was not possible to conclusively show any significant effect of soil type on *E. coli* survival; however the results suggest that there was an association between these parameters. The survival curve for non-sterile sandy loam and clay was similar. For both soil types, *E. coli* concentration remained stable at 4^{o}C, and decreased significantly between T_8 and T_{64} during incubation at 18^{o}C. An increasing trend was observed in sterile sandy loam and clay, but the extent of this increase varied between soils, with higher concentrations consistently extracted from clay at 18^{o}C.

The importance of clay for long-term survival of *E. coli* in soils has been reported previously (Brennan et al., 2010b). As clay soils have a finer texture than sand, they are characterised by greater surface area and relative habitat space (Cools et al., 2001). Clays also have a greater proportion of micropores, which can confer protection to introduced microorganisms. Many studies have shown that pore size is inversely related to the survival of bacteria inoculated to soil (Postma et al., 1990; Heijnen and van Veen, 1991; Wright et al., 1995). Additionally clay holds a net negative charge, which results in nutrient adsorption to the surface of clay particles and provides substrate for the soil biota (Coleman et al., 2004).

Increased survival of *E. coli* in clay soils has been well documented. For example Fenlon et al., (2000) could isolate exogenous *E. coli* for 4 months from clay and loam soils as compared to 8 weeks from sand soils. Similarly, Nicholson et al., (2005) found evidence to suggest that the survival times for *Salmonella, E .coli* O157, *Listeria* and *Campylobacter* were prolonged in beef slurry applied to clay loam grasslands as compared to sandy arable soil. Franz et al., (2008b) showed a negative association between *E. coli* O157:H7 survival and the number of clay particles in manure-amended loamy soils. Mubiru et al., (2000) tested two different silt loam soils, namely Pope and Zanesville, for pathogenic *E. coli* survival. Pope soil contained twice as many clay particles as Zanesville, and death rates for these soils were 0.17 d^{-1} and 0.09 d^{-1}, respectively.

However, other work has shown persistence to be greater in sandy soils, where OM was more influential than soil texture in determining pathogen survival. For

example, Cools et al., (2001) found that *E. coli* concentration was consistently greater in coarse sand as compared to a fine loam soil. This observation was attributed to differences in OM between these soils, which contained 2.4% and 1.1% OM, respectively. Also, Lang and Smith (2007) detected a greater *E. coli* background signal in a sand than in a clay loam. The clay loam contained 30% more OM than the sandy soil, and it was suggested that the higher OM content in clay loam supported greater numbers of antagonistic microorganisms, which resulted in enhanced *E. coli* suppression.

Therefore it is evident that the influence of soil type on pathogen survival is very variable and soil-specific. This experiment showed a significant association between soil type and pathogen survival ($P<0.05$). A greater k-value was obtained for clay as compared to sandy loam, indicating greater persistence of *E. coli* O157 in sandy soil. This may have been due to differences in soil OM and microbial community composition between soil types.

E. coli concentrations increased in sterile sandy loam and clay microcosms. This increase was greater in clay; however, again this could not be confirmed statistically. Better growth may have occurred in clay due to greater nutrient availability, which may have facilitated a more rapid increase in the population.

3.5. Conclusions

The results of this study show that the soil biota has a definite impact on *E. coli* survival. The effect of soil type is more difficult to determine however due to confounding factors such as OM, soil spatial structure and associated habitat space, and microbial diversity. Additionally, the effect of soil biology on *E. coli* survival becomes more apparent at higher temperatures. *E. coli* introduced to cold soils through springtime application of livestock manure may form an environmental reservoir. Based on these results, it is clear that this reservoir will be suppressed as temperatures increase if the native community is intact, as was the case in non-sterile soils in this instance. However, if the native community has been compromised, this reservoir may potentially increase with increasing temperatures due to lack of competition for nutrients and

antagonistic interactions. This shows the importance of a healthy soil microbial community for effective pathogen suppression.

Chapter 4. Interactions between microbial diversity, community structure and pathogen survival in soil

4.1. Introduction

In the previous chapter it was shown that an intact microbial community exerts a strong impact on the survival of *E. coli* O157 (Chapter 3). This model pathogenic organism was capable of growth in sterile soil in the absence of a community, whereas it declined rapidly in non-sterile soil. This observation was attributed to antagonism of native microbiota toward the pathogen in non-sterile soil.

Hence a logical next step in investigating this phenomenon is to determine how pathogen survivability is influenced by differences in extant community complexity, and to determine whether such responses vary according to pathogen type. Both plant and soil models have shown that diversity is associated with functionality, and resistance to disturbance (McGrady-Steed et al., 1997; Naeem and Li, 1997; Girvan et al., 2005). Additionally, resistance to colonisation by invasive species was increased in the presence of a complex plant community (Kennedy et al., 2002). There is also some evidence to suggest that there is an inverse relationship between pathogen survival and community complexity in soil when the pathogen is subjected to distinctly different levels of diversity achieved through a variety of experimental approaches (Toyota et al., 1996; Matos et al., 2005; van Elsas et al., 2007; Ibekwe et al., 2010; Liang et al., 2011). Others have investigated the phenomenon of natural suppression in the context of plant pathogens and have shown that specific organisms rather than collective antagonism exerted by the community as a whole are involved in regulating pathogen survival (Shiomi et al., 1999; Mazzola, 2002; Borneman and Becker, 2007). However, pathogen-specific response to variations across an extensive range of related yet phenotypically-distinct community contexts is unknown.

The first step in carrying out this investigation was to create a wide range of biological contexts in which pathogen responses were quantified. Two commonly used methods of diversity manipulation were considered. Firstly, it has been shown that diversity can be effectively altered in a *constructive* manner using a soil dilution and inoculation approach. In a study by Matos et al., (2005), a microbial suspension was

derived from the rhizosphere of wheat. This suspension was serially diluted and re-inoculated into wheat plants grown in nutrient solution. A reduction in diversity with dilution was confirmed by measuring community function, phenotype, genotype and morphology, thus proving the efficacy of the dilution method. A similar approach was used by Griffiths et al., (2001) to manipulate diversity in entirely soil-based systems. In this case, soil was serially diluted and inoculated into gamma-irradiated soil mesocosms. Dilution factors of 10^2, 10^4, 10^6 and 10^8 were assessed for functional and genetic diversity using CLPP and polymerase chain reaction (PCR)-DGGE methods, respectively. Additionally, agar-media based cultural methods were used to determine the diversity of bacterial and fungal morphology of a cultivable subset of soil microbes across the dilution series, and direct counts of protozoa and nematodes were carried out. These measurements were combined to establish an overall biodiversity index, which clearly demonstrated that diversity within soil mesocosms decreased progressively with increasing dilution. Whereas the soil dilution approach provides a constructive means of manipulating the microbial community, this can also be achieved via *destructive* means. For example, Griffiths et al., (2000) used a differential fumigation technique to investigate the relationship between diversity and function. A study by van Elsas et al., (2007) also employed this method to assess the response of genetically modified *E. coli* O157 to varying levels of community complexity. A range of culture dependent and independent measurements were taken from the manipulated soils, which indicated that the fumigation process had successfully altered the microbial community in the intended manner. A more recent microcosm study applied increasing densities of bile salt to progressively inhibit the native soil community and subsequently assess the persistence of inoculated *E. coli* strains (Liang et al., 2011).

For the purposes of this experiment, we decided to use the dilution approach. This was considered the more appropriate method to use, as unlike the fumigation method it does not select for a particular physiological response and resultant communities are more inclined to be random in constitution (Griffiths et al., 2000), which is arguably a more robust approach in statistical terms since it avoids bias towards fumigation-resistant organisms. Therefore, pathogen response is then measured as a function of a randomly-constituted community in statistical terms.

Soil was therefore serially diluted to achieve differences in community structure, thus creating a nominally wide but consistent gradient in microbial community structure, founded on differences in diversity in the initial inocula. This experimental approach was based on the assumption that rare constituents of the community would be diluted out, whereas abundant constituents would be conserved and carried throughout the series, as described in (Wertz et al., 2006). This biologically-manipulated soil was subsequently incubated to facilitate biomass equilibration, as it was inevitable that soils which received low dilutions not only received a more complex microbial inoculant, but also a greater abundance of microorganisms than those treated with high dilutions. This incubation was required to ensure a comparable microbial biomass across the series, but with different levels of community complexity. Thus any differences observed in pathogen death rates could be attributed solely to the structure of the microbial community. These soils were then inoculated with a suite of model pathogens commonly found in livestock manure, including *E. coli*, *Salmonella* and *Listeria* spp., to determine the impact of community complexity on pathogen-specific decay rates. It was hypothesised that greater pathogen inactivation, measured as increased death rates, would be manifest in the presence of a complex microbial community.

4.2. Materials and methods

4.2.1. Soil sampling and preparation

An arable sandy loam soil was collected from the top 15 cm of Showground Field (X507500, Y235500) at Silsoe Experimental Farm, Cranfield, UK. This soil is classified as Bearsted series, which is described as a Cambisol according to the World Reference Base (FAO, 2006). It is a typical brown earth, described as coarse loamy sand passing to sandstone. Previous analysis has shown that it has a pH of 6.1, total C and N (%) of 1.0 and 0.1, respectively, and a C/N ratio of 10.6 (Jeffery et al., 2007). Soil was taken from the field margin beneath grassy vegetation, which had not received manure or slurry applications for at least 10 years. Fresh soil was sieved to 4 mm and well mixed. Soil WHC was determined by the method described by Franz et al., (2011). Moisture content was then adjusted to 25% of WHC by drying on the bench. This was determined by

oven-drying at 105°C for 24 hours. This soil was sub-sampled and transferred to experimental mesocosms. Each mesocosm consisted of 450 g soil in a one-litre polypropylene pot with a screw top lid. A 7 mm diameter high-efficiency particulate air (HEPA) filter was secured into the top and sealed above and below the lid using silicon sealant to facilitate gas exchange. Mesocosms containing soil were then sent to Isotron, Swindon, where they received a single dose of gamma irradiation at 25 kGy.

4.2.2. Community structure manipulation

A community structure manipulation procedure was carried out by inoculating the irradiated soil with selected serial dilutions of field soil.

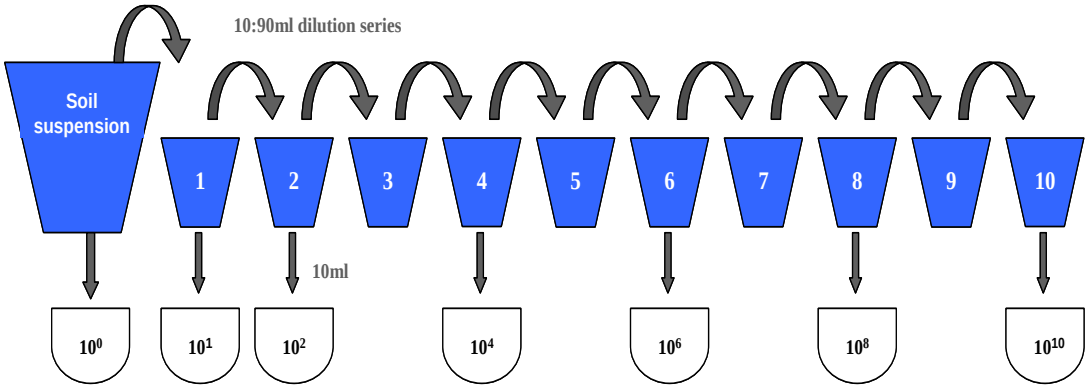


Fig. 4.1 Establishment of soil dilution series

The series was created by placing a 25 g portion of field soil into 100 ml of sterile ¼ strength Ringers solution in sterile bottles, denoted a 10^0 (i.e. one-fold) dilution. This soil slurry was mixed on an orbital shaker at 140 rpm for 15 minutes. Ten ml of the 10^0 slurry was transferred to 90 ml of sterile ¼ strength Ringers solution, and shaken as before to create a 10^1 dilution. This process was repeated to create dilutions to a factor of 10^{10}. The irradiated mesocosms were then inoculated with 10 ml of selected dilutions, i.e. 10^0, 10^1, 10^2, 10^4, 10^6, 10^8 and 10^{10} (Fig. 4.1). Additionally, irradiated mesocosms were inoculated with 10 ml sterile ¼ strength Ringers solution alone, hereafter denoted as 'irradiated control'. Inoculation was carried out under aseptic conditions in a laminar flow cabinet. The final soil moisture content attained following this procedure was 30% of WHC. Four independent dilution series of this nature were

produced, providing four replicates of each of 7 dilutions plus a single irradiated control (i.e. a total of 32 mesocosms). Mesocosms were then incubated in the dark at room temperature for a period of 322 days.

Microbial biomass was measured on a regular basis by subjecting sub-samples of soil from each mesocosm to CFE at 14, 70, 112 and 154 days post-establishment, and SIR after 210 and 320 days. These techniques are described in Section 2.2 and 2.3, respectively. Soil in the mesocosms was mixed during the incubation period via regular gentle agitation approximately every 2 weeks. Moisture content was also maintained at a constant level by regular weighing and addition of sterile water as required, taking into account any loss in weight incurred by the removal of mesocosm soil over the sampling period. When microbial biomass had been confirmed as convergent across all treatments, mesocosms were sub-sampled and phenotypic community structure determined using the PLFA technique, as described in Section 2.4.

4.2.3. Pathogen inoculation and survival analysis

Following equilibration of biomass across the dilution series, mesocosm soil was used to establish microcosms for pathogen inoculation and subsequent survival analysis. Microcosms consisted of 5 g soil portions in sterile polypropylene vials (40 ml), which were covered with Parafilm to ensure constant moisture status, and prevent further microbial incursion, whilst allowing gaseous exchange. Microcosms were maintained at 10^{o}C throughout. Inoculum cultures were grown and inoculated according to Section 2.1.3. Microcosms were independently inoculated with 100 µl of either *S.* Dublin, *L. monocytogenes*, *E. coli* Lys 9 or *E. coli* O157 cultures, containing approximately 10^{8} cells as determined according to the method described in Section 2.1.3, thus delivering 10^{7} cells g^{-1} soil. This resulted in final soil moisture of 34% of WHC. Microcosms were sampled destructively immediately after inoculation (hereafter denoted T_0) and 2, 4, 8, 16, 32, 75 and 160 days post-inoculation (hereafter denoted T_2, T_4, T_8, T_{16}, T_{32}, T_{75} and T_{160}). Enumeration was carried out as described in Section 2.1.4.

4.2.4. Statistical analysis

One-way ANOVA was used to compare biomass concentrations. PLFA profiles were subjected to PCA, as described in Section 2.5.2. Exponential decay curves were fitted to data of average triplicate CFU counts as described in Chapter 2.5.3, to calculate pathogen death rates. The resultant death rates were then plotted in turn against soil dilution and the first four PC scores to determine if there was any correlation between pathogen survival and microbial diversity or community structure, respectively. The underlying assumptions of statistical tests were checked and all statistical analyses were carried out using Statistica (Version 10).

4.3. Results

4.3.1. Microbial biomass

Biomass ranged between 0 to 50 μg C g^{-1} 14 days post-inoculation with the soil suspension, which was low as compared to subsequent sampling dates. Biomass consistently increased during the incubation period. CFE showed that biomass was equitable after 154 days, and this was confirmed by SIR at 240 days and 322 days. It was decided to sample approximately 1 year post-inoculation, as this was the time taken for biomass to equilibrate in a similar manipulation previously (Griffiths et al., 2001). There were no significant differences in biomass between treatments at any stage ($P>0.05$), measured by CFE (Fig. 4.2a), and SIR (Fig. 4.2b), indicating that biomass was comparable between dilutions prior to pathogen inoculation for survival analysis.

4.3.2. PLFA analysis

In the PCA of these profiles, the first four PCs accounted for 58% of the variation; beyond PC4, less than 6% further variance was accounted for by each component. Significant effects of soil inoculum dilution upon the phenotypic structure of the resultant microbial communities were apparent for PC1 ($P<0.05$) and PC3 ($P<0.01$) only. There were no significant treatment effects for PC2 or PC4 ($P>0.05$). Ordination of PC1 against PC3 revealed no simple trend between treatments within this PC space, but there was a trend for the lower dilutions (i.e. 10^0 and particularly 10^1 and 10^2) to be

distinct from the higher dilutions in PC3, associated with an overall trend relating to increasing dilution in this third PC (Fig. 4.3a). In addition, dilutions 10^6 and 10^8 were significantly separated from the other treatments with respect to PC1 (Fig. 4.3a). Ordination of the loadings showed that neither PC was dominated by particular PLFA types, rather being founded upon broadly similarly weighted combinations of a number of PLFAs (Fig. 4.3b). PLFAs were labeled numerically to aid visualization on the PC plane (Table 4.1).

4.3.3. Pathogen decay rates – curve fitting

All pathogens displayed broadly similar survival characteristics and their concentrations in all microcosms declined exponentially (Fig. 4.4). There was evidence of some differences in response behaviour between treatments mid-phase, reflected by differences in k-values (Table 4.2). The exponential decay function was a significant fit for all pathogens and treatments ($P<0.05$). Overall pathogen decline during the experimental period was similar, and a value of approximately 99% decline, relative to the number of cells initially added, was recorded for all pathogens and treatments.

When the relationships between dilution treatments and k-values were compared (Fig. 4.5 and 4.6), the irradiated control was isolated from the other treatments since it did not form a coherent part of the dilution series. Three different classes of behaviour were observed: (i) an apparently linear negative relationship between dilution treatment and death rate for *S.* Dublin (Fig. 4.5a) and *E. coli* Lys 9 (Fig. 4.5c), which were strong ($P<0.05$), and marginal ($p=0.10$) respectively; (ii) an overall lack of effect with an idiosyncratic response, such as for *L. monocytogenes*, where the 10^6 dilution treatment showed a significantly ($P<0.05$) greater death rate than for all other dilutions (Fig. 4.5b); (iii) no effect of dilution upon death rate such as for *E. coli* O157 (Fig. 4.5d).

With respect to principal components and decay rate, there was a strongly significant linear relationship between PC3 and k-value for *S.* Dublin (Fig. 4.6a; $P<0.0001$) and *E. coli* Lys 9 (Fig. 4.6c; $P<0.05$). There was an idiosyncratic effect of dilution 10^6 for *L. monocytogenes* for PC3 ($P<0.05$; Fig. 4.6b), which was also observed for PC1, 2 and 4 (Appendix 1A, 1B and 1C, respectively). There were no other apparent relationships between k-value and PCs1-4 (Appendix 1A, 1B and 1C, respectively).

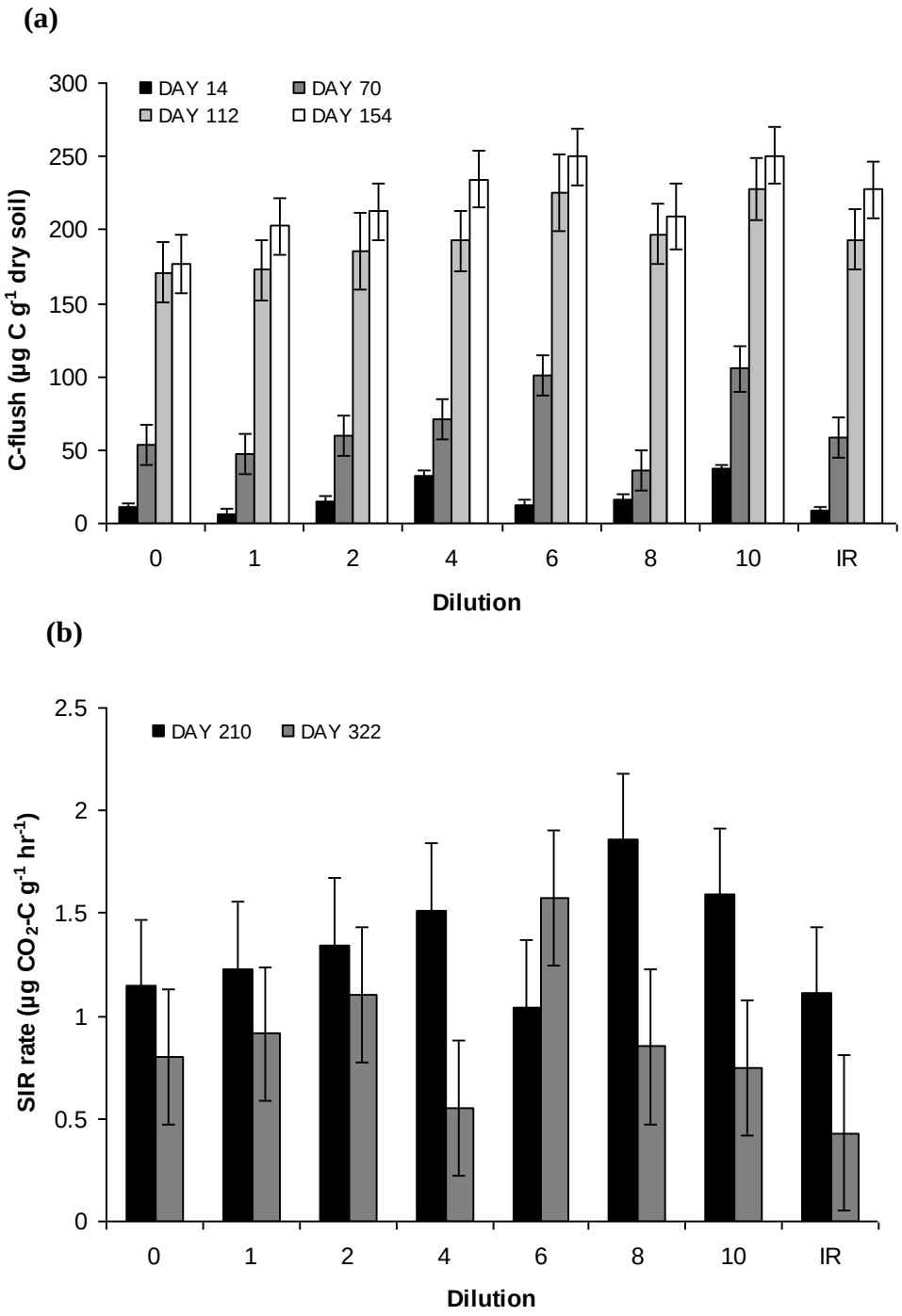


Fig. 4.2 Microbial biomass carbon across soil dilution series as measured by (a) CFE at Day 14, 70, 112 and 154 post-inoculation and (b) SIR techniques at Day 210 and 332 post-inoculation. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4). P>0.05 for within time effects in all cases

Table 4.1 Reference numbers used for PLFAs, shorthand and systematic names (c.f. Fig. 4.3)

PLFA ID, shorthand and systematic name					
ID	**Shorthand**	**Systematic name**	**ID**	**Shorthand**	**Systematic name**
1	**12:0**	**Me. dodecanoate**	25	PLFA_14	unidentified
2	PLFA_1	unidentified	26	PLFA_15	unidentified
3	PLFA_2	unidentified	27	PLFA_16	unidentified
4	**13:0**	**Me. tridecanoate**	**28**	**i17:0**	**Me. 15-methylhexadecanoate**
5	PLFA_3	unidentified	29	PLFA_17	unidentified
6	PLFA_4	unidentified	30	PLFA_18	unidentified
7	PLFA_5	unidentified	**31**	**17:0c**	**Me. Cis-9,10-methylenehexadecaonate**
8	PLFA_6	unidentified	**32**	**17:0**	**Me heptadecanoate**
9	PLFA_7	unidentified	33	PLFA_19	unidentified
10	**14:0**	**Me.tetradecanoate**	34	PLFA_20	unidentified
11	PLFA_8	unidentified	35	PLFA_21	unidentified
12	PLFA_9	unidentified	**36**	**18:2w6c**	**Me. cis-9,12-octadecadienoate**
13	**i15:0**	**Me. 13-methyltetradecanoate**	**37**	**18:1w9c**	**Me. cis-9-octadecanoate**
14	**a15:0**	**Me. 12-methyltetradecanoate**	**38**	**18:1w9t**	**Me. trans-9-octadecanoate**
15	PLFA_10	unidentified	39	PLFA_22	unidentified
16	**15:0**	**Me. Pentadecanoate**	**40**	**18:0**	**Me. octadecanoate**
17	**2-OH 14:0**	**Me. 2-hydroxytetradecanoate**	41	PLFA_23	unidentified
18	**3-OH 14:0**	**Me. 3-hydroxytetradecanoate**	42	PLFA_24	unidentified
19	**i16:0**	**Me. 14-methylpentadecanoate**	43	PLFA_25	unidentified
20	PLFA_11	unidentified	**44**	**19:0c**	**Me. cis-9,10-methyleneoctadecanoate**
21	**16:1w7c**	**Me-cis-9-hexadecanoate**	**45**	**19:0**	**Me. nonadecanoate**
22	PLFA_12	unidentified	**46**	**20:0**	**Me. eicosanoate**
23	PLFA_13	unidentified	47	PLFA_26	unidentified
24	**16:0**	**Me. hexadecanoate**	48	PLFA_27	unidentified

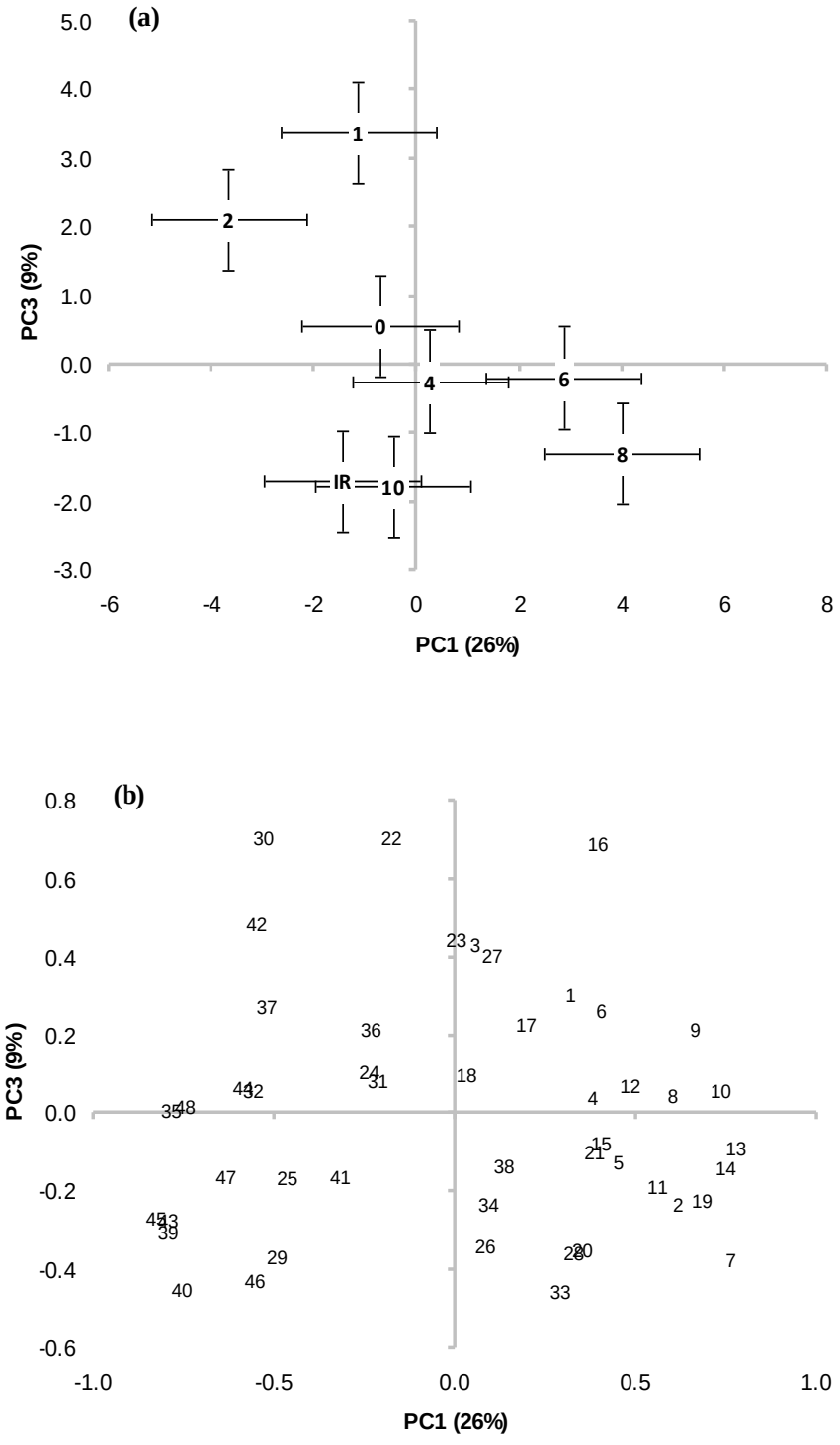
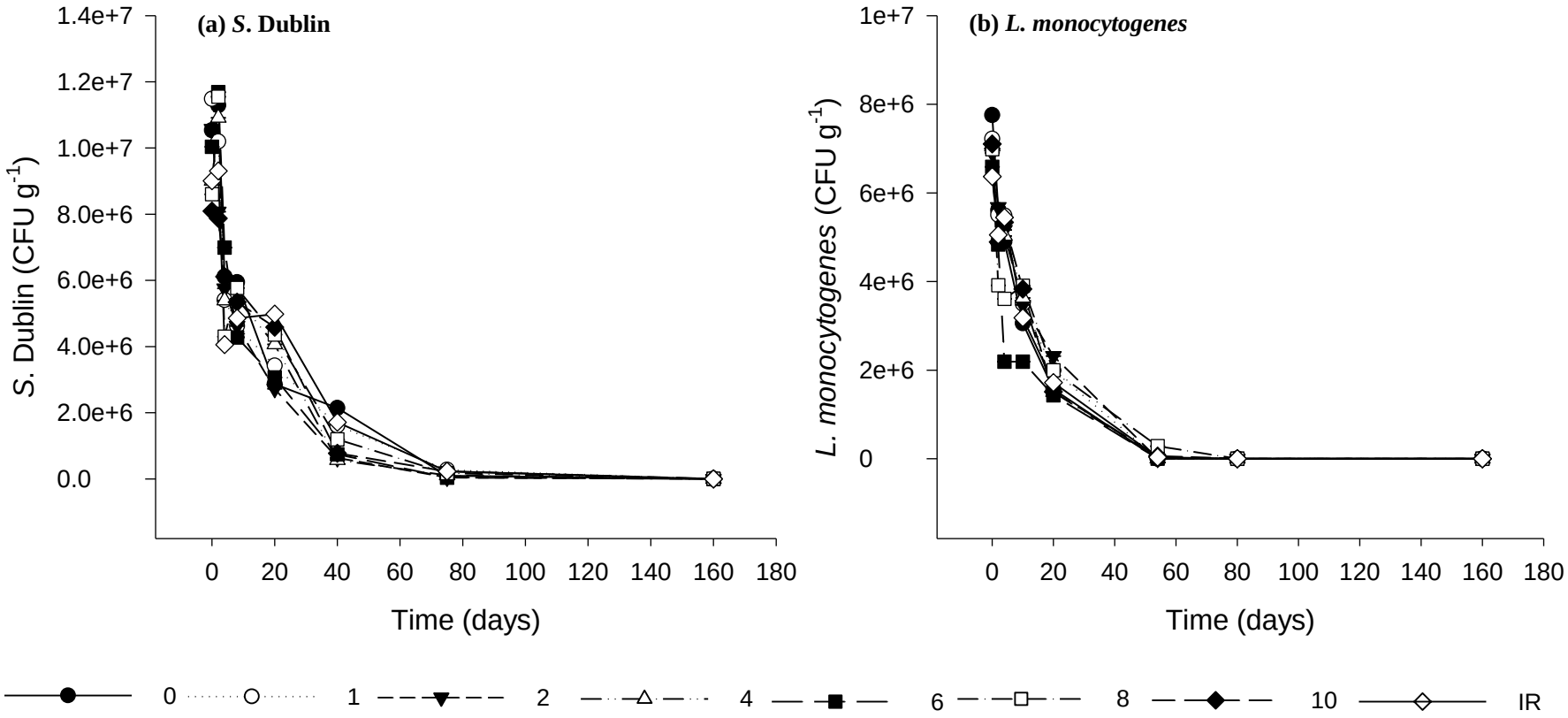


Fig. 4.3 (a) PC plot showing ordination of first and third PC scores from average PLFA profiles across soil dilution series and (b) corresponding PC loading plot showing PLFAs responsible for PC discrimination. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4). See Table 4.1 for PLFA identification


(a) S. Dublin
S. Dublin (CFU g-1)
1.4e+7
1.2e+7
1.0e+7
8.0e+6
6.0e+6
4.0e+6
2.0e+6
0.0
0
20
40
60
80
100
120
140
160
180
Time (days)
(b) L. monocytogenes
L. monocytogenes (CFU g-1)
1e+7
8e+6
6e+6
4e+6
2e+6
0
0
20
40
60
80
100
120
140
160
180
Time (days)
0
1
2
4
6
8
10
IR

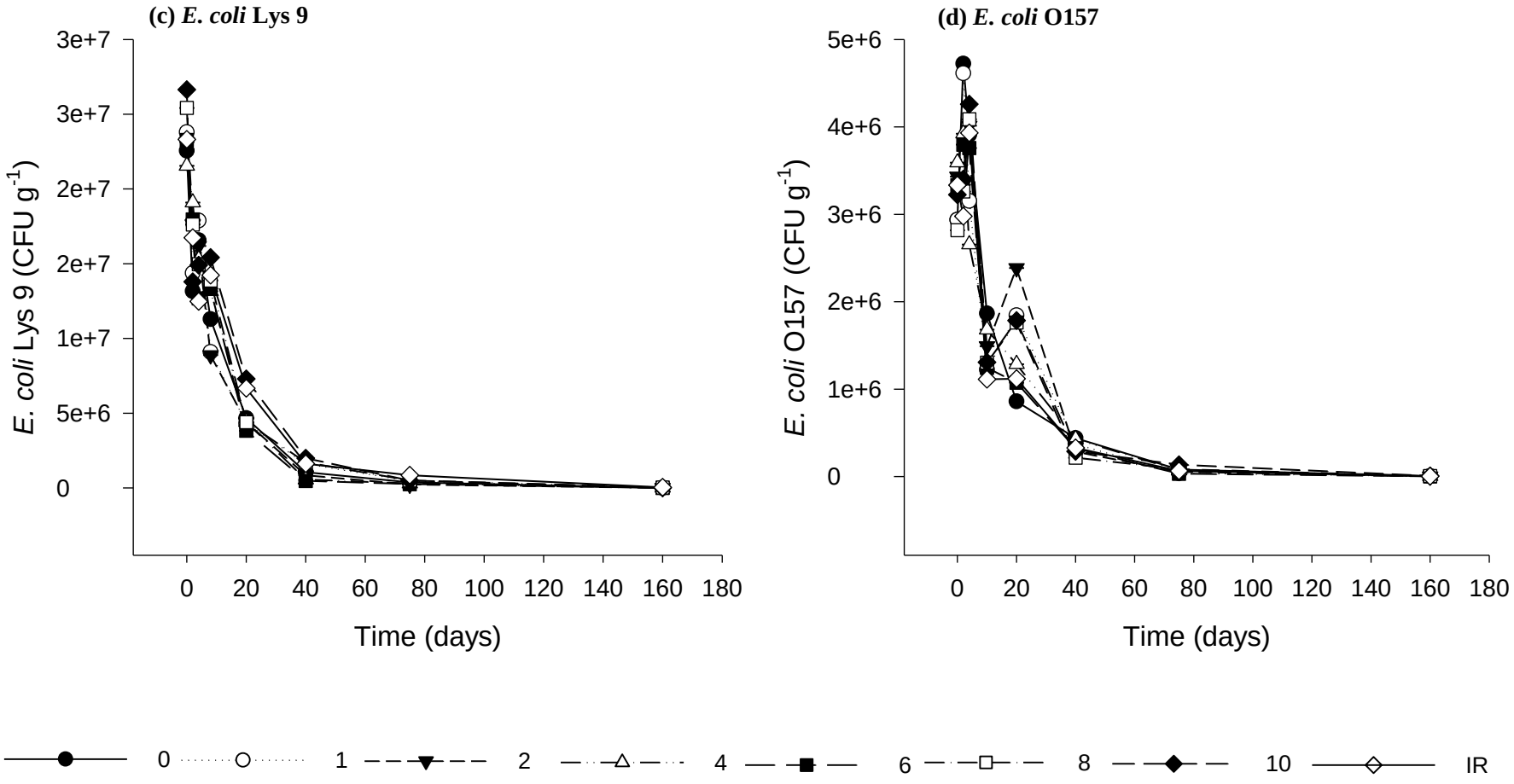


Fig. 4.4 Survival of (a) *S*. Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157 in across soil dilution series. Data represent CFU g^{-1} soil (dry weight) ± standard error (n=4). 0-10 = dilutions 10^0-10^{10}, IR = irradiated control. Note that error bars fall within confines of treatment symbols

Table 4.2 Decay rates of pathogens introduced into soils

	k-values (days^{-1})*			
Dilution	***S*. Dublin**	***L. monocytogenes***	***E. coli* Lys 9**	***E. coli* O157**
0	0.08±0.02	0.09±0.01	0.08±0.01	0.06±0.01
1	0.13±0.02	0.07±0.01	0.10±0.01	0.05±0.02
2	0.10±0.02	0.06±0.01	0.10±0.01	0.04±0.01
4	0.06±0.02	0.07±0.01	0.07±0.01	0.06±0.01
6	0.09±0.02	0.17±0.04	0.09±0.01	0.07±0.02
8	0.05±0.02	0.06±0.02	0.08±0.01	0.04±0.02
10	0.04±0.01	0.07±0.01	0.06±0.02	0.05±0.02
IR	0.04±0.02	0.06±0.01	0.07±0.01	0.07±0.02

*Exponential decay model significantly fit curves for all pathogens and treatments ($P<0.05$)

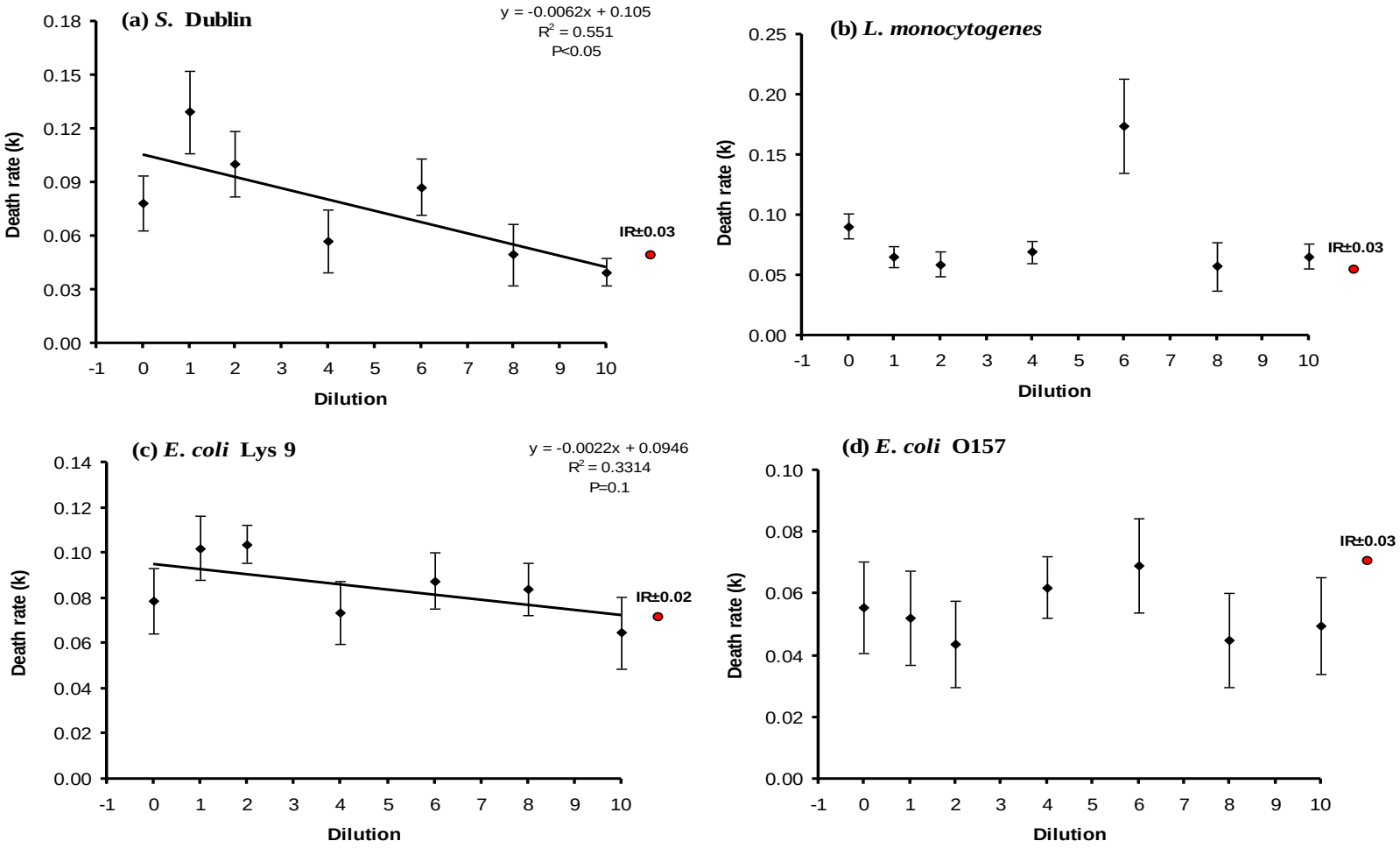


Fig. 4.5 Relationship between pathogen death rate and soil dilution treatment for (a) *S.* Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4)

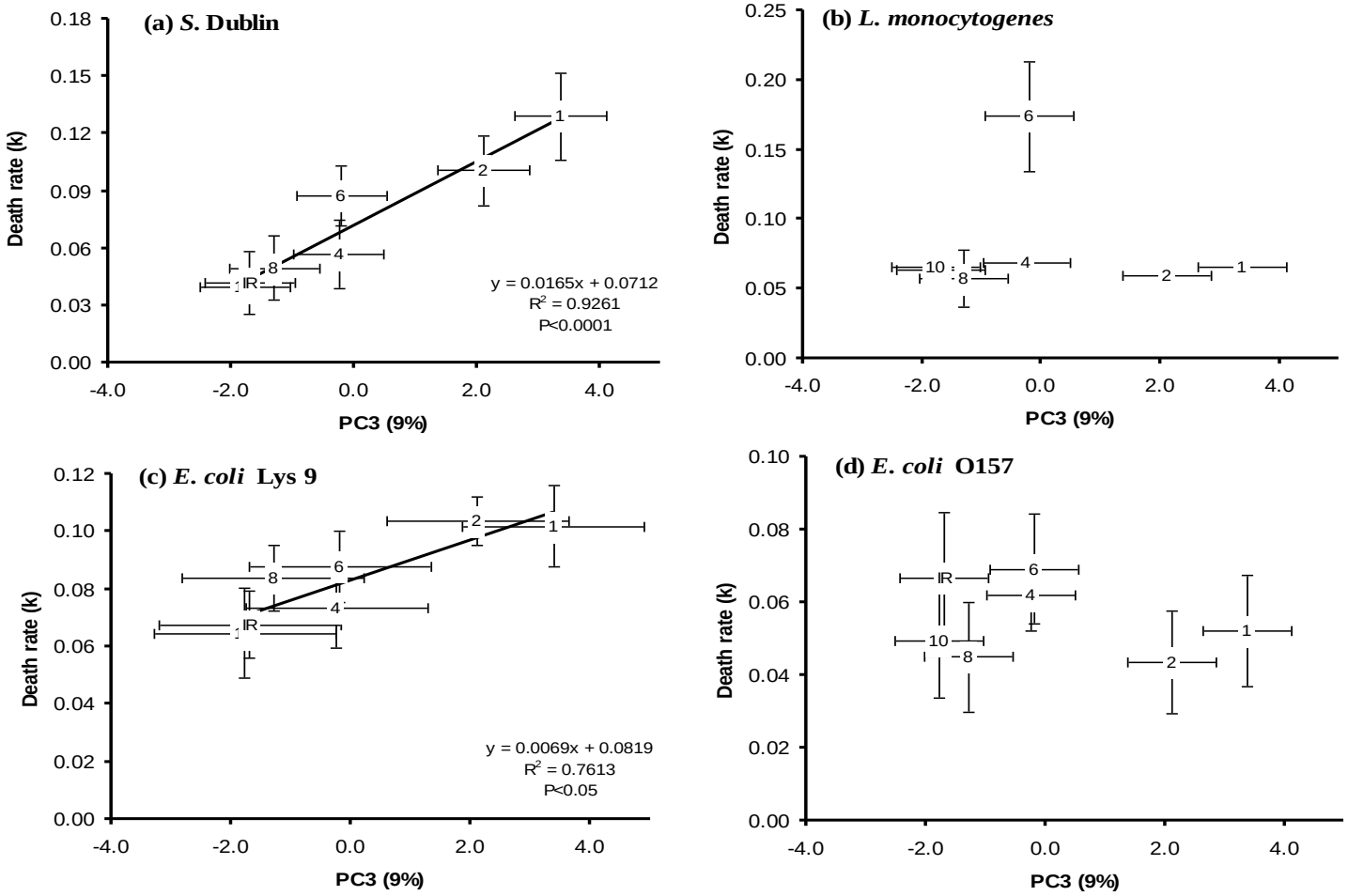


Fig. 4.6 Relationship between pathogen death rate and third principal component for (a) *S*. Dublin (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4), which fall within confines of symbols for some treatments

4.4. Discussion

The aim of this experiment was to assess the relationship between microbial community structure, and nominally microbial diversity, and the survival of pathogens typically found in organic wastes. It was hypothesised that there would be an inverse relationship between community complexity and pathogen survival. To test this hypothesis, it was required to create a range of community contexts, and this was done using a soil dilution approach. PC analysis showed that this approach was successful in terms of generating the intended treatment range, i.e. a series of contrasting phenotypic structures across the dilution series which showed some coherence in the way they changed. This was evident, particularly in the case of PC3, which certainly separated higher dilutions from lower dilutions. However, this separation wasn't as strong as expected in the case of PC1, and significance of PC scores indicated that community differences between dilutions were subtle. For instance, PC1 and PC3, which collectively accounted for 35% of the variability between dilution treatments, fell just within the 5% significance level ($P<0.05$), whereas PC2 and PC4 were not significant. Also, the PLFA loadings associated with the significant PCs showed that no dominant PLFAs could account for the variation in community structure between dilution treatments, and hence the differences between these phenotypes across the dilution series was not founded on simple differences between treatments. This subtle difference in microbiological context between treatments complements the circumstance in the study in the previous chapter when the differences were at the other extreme.

It is notable that despite extensive dilution to a factor of 10^{10}, differences in community composition were subtle at the end of the incubation period. According to Torsvik and Øvreås (2002), 1 g of soil can potentially contain 10 billion microorganisms. Other work has estimated that 1 g can contain 10^9 cells, comprising 10^6 different taxa (Curtis and Sloan, 2005; Gans and Dunbar 2005). Based on this assumption, dilution to 10^{10} should therefore have only received tens of cells, and very limited diversity. Nevertheless, the resultant phenotype developed in a broadly similar fashion, such that only PC1 and PC3 were significant. The fact that each treatment was created in the context of the same soil type may potentially account for the observed similarities. It has been found that microorganisms alter their cell membranes, according to prevailing environmental conditions (Kaur, 2005). Therefore, the soil communities

within dilution treatments may have converged as the microbial inoculum adapted to prevailing soil conditions. There is also evidence to suggest that the complex of edaphic factors of the soil governs the eventual community structure, regardless of what was added initially (Griffiths et al., 2008). This study manipulated the microbial community by inoculating gamma-irradiated soils with a complex microbial mixture derived from sand and clay loam soils. It was found that when the same communities were inoculated into different soils, they developed differently, whereas when different communities were inoculated to the same soil, they developed in similar fashion. Here, a single soil type was inoculated with a range of communities that differed in structure and complexity. Much like Griffiths et al., (2008), these communities developed in the same way during incubation. Therefore, it is possible that soil structure and physico-chemical composition directed the development of the microbial community.

Irradiated control soils were established to ensure that contamination of treated soils did not take place, and that community development was not influenced by an atmospheric microbial signal. However the irradiated soils tested positive for biomass by CFE and SIR on all sampling dates, and had a phenotypic profile similar to other dilutions at the end of the incubation period, indicating that these soils were not sterile, as intended. Soils were exposed to the atmosphere during the inoculation procedure and subsequent sub-sampling which may have resulted in contamination. Alternatively, the soils may not have received a sufficiently high dose of gamma irradiation to effectively kill resistant microbial spores. However, increasing the irradiation dose can alter soil physico-chemical composition, and thus a potential trade-off exists between sterility and resultant integrity of soil structure (McNamara et al., 2003). Despite the fact that these soils were possibly influenced by microorganisms other than those added in the inoculum, this does not compromise the interpretation of results. The primary goal was to create soils with different levels of community structure, with an inferred gradient in biodiversity. The fact that the irradiated control soil ordinates with the highest dilution in the series, 10^{10}, and lower dilutions ordinate consistently according to PC3, provides evidence that this goal was achieved.

Nonetheless, the phenotypic similarities between low and high dilutions as reflected by lack of separation in PC1 and limited overall significance of PC scores are curious. While the phenotype gives an insight into the overall structure of the

community, it does not provide any information at species level. These dilutions may contain similar structures but very different species composition. Therefore a genetic characterisation of these soils would have been useful, to discriminate species differences between treatments. Additionally, analysis of the temporal development of both the phenotype and the genotype would have provided a more comprehensive assessment of the community in this case.

It was hypothesised that pathogen survival would vary between communities, and that pathogens would persist in soils at the higher end of the soil dilution series, due to a decrease in the likelihood of antagonistic interactions with the native community, and a corresponding increase in the availability of functional and physical niche space. While an effect of the soil community on pathogen death rates was detected, this was variable and context-dependent. The PLFA technique used to profile the soil community does not provide any direct information on the overall diversity (i.e. number of distinct organisms) within these samples, but rather provides a quantifiable description of the resultant phenotypic (Frostegård et al., 2010). Nucleic acid profiling provides a more direct measure of (genetic) diversity *per se*, but this was not adopted in this study. It was hypothesised that pathogen response would be stronger to the community phenotype than the genotype, as the phenotype represents the environmental expression of the genotype. Therefore the pathogens are directly exposed to the phenotype upon addition to the soil, and not the genotype, which is arguably a functional abstraction of the community. The phenotype was thus considered to provide a more appropriate and operationally relevant measure of the community in this case. It was assumed that an inherent diversity effect would be manifest across treatments based on the logic of the dilution approach adopted. Some pathogens did show evidence of a diversity effect, indicated by the linear relationship between the extent of soil dilution and pathogen decay. This linear response provides support for the hypothesis of an inverse association between diversity and pathogen survival. However, a considerably stronger effect was observed in relation to PC scores, notably for the pathogens which also showed a relationship to dilution *per se*, particularly with respect to PC3, suggesting that the soil community structure, rather than the inherent diversity, plays a more important role in regulating pathogen decay.

The effect of community complexity on pathogen survival has been shown previously, notably for *E. coli* strains. These studies have consistently reported a strong inverse relationship. For example, soil exposed to progressive fumigation intensities resulted in enhanced survival of a genetically modified strain of *E. coli* O157 (van Elsas et al., 2007). Similarly, Ibekwe et al., (2010) showed that fumigation as a soil management technique resulted in simplification of the microbial community, which was associated with enhanced survival of *E. coli* O157. Work by Liang et al., (2011) used different concentrations of bile salt to alter community composition and achieve different levels of complexity. It was shown that *E. coli* survival was positively correlated with bile salt concentration, demonstrating the ability of this pathogen to persist when niches for space and nutrients are made available. However, in the current study, although signals of a negative diversity, and specifically complexity, effect were detected, pathogen survival characteristics were more variable between treatments and pathogen types. One factor that may account for this variation is the occurrence of interactions that may have benefited the pathogen. For instance, the communities that established within the dilution series may have randomly included organisms that were capable of breaking down complex organic materials to simpler forms that were then available for uptake by the pathogen, thus prolonging survival. Nevertheless, this study has effectively compared pathogen survival behaviour in the context of an extensive range of communities, and has shown that subtle differences in community complexity are sufficient to cause differences in survivability. It has also shown that the community effect differs between pathogen types. Differential survival between *E. coli* strains in soil has been reported previously, attributed to differences in cell structure and chemistry, and nutrient utilisation capability (Topp et al., 2003); however, comparative work between pathogen types is lacking. Therefore future studies investigating the role of the community in pathogen decay should not rely on *E. coli* to predict the survival characteristics of other organisms.

Pathogen behaviour was not regulated by specific phospholipid types (and nominally microbial groups), but rather a complex combination of a range of PLFAs, demonstrating that in this instance, survival was primarily influenced by the *overall community context*. This corresponds to a general rather than specific suppressive effect. General suppression is related to competition with the total community for nutrients and

niche space, whereas specific suppression is caused by negative, notionally specific, interactions between particular organisms (van Bruggen and Semenov, 2000). In light of these results, subtle changes to the cohort of microorganisms present in the soil may have a very significant impact on pathogens capability to persist.

4.5. Conclusions

This experiment has provided evidence that pathogen survival can be influenced by the diversity of the microbial communities which the pathogens encounter, but that the phenotypic structure of the community has a stronger effect. It has also shown that such effects on survival can manifest where differences in the community phenotype are subtle. In some cases, there was an inverse relationship between diversity, particularly community complexity, and pathogen decline. The effect of the community on survival characteristics differed between pathogen types, thus demonstrating a context dependency on such effects. This is important as it highlights the need to include a range of pathogens in studying such phenomena in that there is no apparent simple generalisation. The inference is that pathogen survival was associated with generally-found microbial rather than any specialist-based suppression. This suggests that manure-derived pathogens are regulated by interactions with the total microbial consortium, rather than by antagonism from specific microbial groups.

Chapter 5. Prescription of soils in order to determine impact of microbial community phenotype on pathogen survival

5.1. Introduction

The soil microbial community is typically sensitive to changing environmental conditions, and consequent shifts in community structure could influence the survival of introduced pathogens. Thus far, we have investigated the effect of experimental manipulation of the soil microbial community on pathogen survival. It has been shown that pathogens can persist and grow where biology is absent. In contrast, soil dilution and resultant simplification of a single community had a subtle but detectable impact on overall community structure and associated pathogen decay. We will now focus on profiling natural soils derived from the field with contrasting edaphic and biological contexts, with a view to studying pathogen decay in such circumstances. Such soils have intact communities which are the product of complex interactions between soil type, prevailing environmental conditions, predominant land-use and land-management strategies. Many studies have been carried out to investigate the impact of land-use on microbial community structure (Hartmann et al., 2006; Esperschütz et al., 2007; Jangid et al., 2008); however the aim of this experiment was to extend this knowledge further and to investigate consequent effects of such communities associated with different land-uses on pathogen survival.

Land-use can influence soil pH, mineralizable carbon and N, and nutrient availability (Jangid et al., 2008). It can also dictate the arrangement of soil pore space, which regulates air and water movement throughout the soil matrix. Therefore, different aspects of land-use will influence the structure of the soil microbial community in a variety of ways. Such aspects include the predominant management strategy, application of organic amendments, mineral fertilisers and cropping and tillage practices. Lauber et al., (2008) suggested that changes in soil edaphic factors stimulated by land-use were primarily responsible for variation in soil microbial composition.

Jangid et al., (2008) showed that the intensity of land-use can be an effective predictor of community structure and diversity. Their work compared the microbial

structure of cropland, pasture and forest soils. Complex community configurations were observed in pasture soils, and simpler communities were observed in cropland and forest soils in comparison. Pasture represented intermediate disturbance in this instance, whereas forest and cropland were associated with low and high disturbance, respectively. Another factor that exerts a strong impact on the soil microbiota is the length of time under a particular management, and so-called 'legacy effects' can become apparent following a change in land-use. The legacy effect describes the residual impact of previous management on the soil community. For example, Buckley and Schmidt (2001) found that community structure was similar in soils subjected to long-term management; however soils which received the same management over a short-term period fostered distinctly different communities, which were assumedly associated with the preceding management regime. The quality, quantity and diversity of substrates added to the soil, in either organic or mineral fertiliser form, will also impact on the structure, diversity and function of the soil microbiota. It has been shown that soil C/N ratio and relative amounts of humic substances can account for variation in bacterial community structure at a genetic level (Lejon et al., 2007). Microbial function and contribution to decomposition of OM is partially regulated by substrate availability (Hernández and Hobbie, 2010).

In addition, Degens et al., (2000) found a significant positive linear relationship between catabolic evenness of the microbial community and organic carbon reserves, suggesting that the preservation of soil organic carbon stocks is a crucial element to maintaining microbial diversity. Other work has shown that contrasting communities can develop in the presence of the same crop type, as a result of different practices associated with organic and conventional management (Esperschütz et al., 2007). Such studies strongly suggest that land-management regimes, which regulate the quantity and quality of soil OM, have site-specific impacts on soil microbial biomass and community structure.

The type of crop species planted may also exert a rhizosphere effect on community structure, due to the exudation of a variety of extracellular polysaccharides and species-specific variation in the rate of root development (Garbeva et al., 2004b). However the influence of the rhizosphere on the development of microbial diversity is debatable and contrasting results can be found in the literature. Some studies have

shown differences in communities associated with specific plant species and associated rhizosphere conditions e.g. Smalla et al., 2001; Costa et al., 2006; Maul and Drinkwater, 2010; Yin et al., 2010. However, other work has demonstrated limited rhizosphere effects. Kielak et al., (2008) used molecular techniques to reveal significant variation within the microbial community between bulk and rhizosphere soil; however further experimental work demonstrated little effect of plant species or diversity on rhizosphere community structure. Differences in rhizosphere effects between studies were attributed to the overriding influence of soil type and soil management history on microbial community development.

The aim of this experiment was two-fold; firstly, to prescribe soils from different land-uses that possessed contrasting physico-chemical and microbial community composition, and secondly to identify predominant influential factors regulating pathogen decay in these soils. Initial soil prescription was done using a novel screening technique based on PC analysis of soil PLFA profiles, taking land-use and selected physico-chemical soil properties into account. This PLFA-PCA screening method has not been used previously for the purposes of soil selection. For the reasons discussed above, it was assumed that soils from different land-uses would possess highly contrasting communities. The purpose of this screening process was to identify an appropriate suite of soils for subsequent application in pathogen survival analysis. It was hypothesised that biological factors rather than physico-chemical properties would be more important in regulating pathogen decay. This chapter then reports the first stage of the experiment which involved PLFA profiling of soils from a wide range of sites, and the prescription of suitable soils for pathogen survival analysis.

5.2. Materials and methods

5.2.1. Site selection

The first stage of this experiment was to carry out a desk study in order to identify suitable sites for sampling, where contrasting microbial communities would be anticipated. This was achieved by examining existing maps of Teagasc sites, and supplementary information on soil type, current land-use and land-use history where

available. A total of 39 sites were selected for sampling, based on contrasting land-use, soil type and management regime. These were categorised into 3 land-use classes, namely grassland, arable and wood. These contained 12, 10 and 17 of the total suite of 39 soils, respectively. Similarly soils of different textural classes were divided into sand silt and clay dominated, based on hand texturing analysis. These classes contained 12, 15 and 12 soils, respectively. Sites were located at Johnstown Castle (Co. Wexford), Moorepark (Co. Cork), Oak Park (Co. Carlow) and Kildalton (Co. Kilkenny). Further details of site location are provided (Table 5.1). A stratified design was adopted: sites consisted of a single uniform field which was divided into 3 sections, and each section served as a replicate within that site. Replicate sections were sampled by Dutch auger (Eijkelkamp, The Netherlands) using a W-of-best-fit transect design. Multiple cores (approximately 20) were taken from the top 15 cm of soil across the W transect from each section, and were combined to yield a composite sample.

5.2.2. Preliminary soil screening

Soils from all 39 sites were stored in loosely tied plastic bags at 4^{o}C until use. They were then homogenised and sieved to 4 mm. Soil sub-samples were frozen overnight at -80^{o}C, followed by freeze-drying at -57^{o}C. Aliquots of approximately 5-10 g freeze-dried soil were weighed out and screened for community composition by PLFA as outlined in Section 2.4. Soils were also tested for pH, % OM and texture, as described below, to give an indication of physico-chemical composition.

5.2.2.1. Soil pH

Soil pH was determined using an automated Aqualyser pH meter (Envirotech, UK). The pH meter was calibrated using pH 4 and pH 7 buffer solutions. Aliquots of 10 g fresh soil were suspended in 25 ml deionised water, and the electrode was placed into the suspension. The sample was stirred and when the pH of the sample had stabilised, the reading was recorded. The electrode was then washed in deionised water before processing further samples.

5.2.2.2. Soil OM

Soil OM was determined using the loss on ignition technique (Davies et al., 1973). Briefly, soil was air dried and sieved to 2 mm. Soil was then oven-dried overnight at 105^{o}C to remove moisture. Aliquots of 4 g oven-dried soil were weighed into crucibles and were then placed into a muffle furnace (Gallenkamp, UK) for 16 hours at 500^{o}C. OM was calculated according to the following equation:

(Weight after drying at 105^{o}C – Weight after ashing at 500^{o}C)*100

5.2.2.3. Soil texture

Soil texture was determined using the hand test (DEFRA, 2010). Approximately 10 g soil was gradually moistened and kneaded between fingers and thumb until soil exhibited maximum cohesion. Texture was assigned according to a soil textural class identification chart (DEFRA, 2010).

5.2.3. Data analysis and visualisation

PC analysis was carried out on mol % values derived from PLFA profiles for these soils (Statistica, Version 10). First and second components were then plotted and labelled according soil ID, land-use, texture, pH and OM using mean PC scores, to visualise differences in community structure. Data was also aggregated according to land-use and texture, and a one-way ANOVA was used to test for main effects associated with these characteristics.

5.3. Results

Details of site location, physico-chemical parameters and PC scores derived from PLFA profiles are provided (Table 5.1). A wide range of values were obtained for both pH and OM (Table 5.1). The first four PC scores were highly significant ($P<0.001$) and collectively accounted for 61% of the variation in community composition between sites (Table 5.1). PLFAs were labelled numerically to aid visualisation on PC plots (Table 5.2).

Soils were plotted according to community composition (Fig. 5.1a). The degree of separation between soils visually represents the level of dissimilarity between soil communities. For example, soil from JC1 in the bottom quadrant of the PC plane is by definition highly dissimilar to soil from OP3 in top right quadrant. Conversely, soils that cluster together possess similar phenotypic community composition. For example, KD5 and KD6, collected from adjacent fields at Kildalton, occur very close together on the PC plane, demonstrating that these soils share similar community phenotypes. This PC plot shows that soils are notably dispersed across the PC plane, indicating that these soils possess a broad range of inherently contrasting communities. Similarly the corresponding PC loadings plot shows that a wide range of PLFAs contribute to the differences detected between the range of phenotypes (Fig. 5.1b).

Average PLFA profiles for each land-use class were used in PC analysis. This analysis showed that the first principal component accounted for 32% of the variability between soil communities associated with different land-use classes; however one-way ANOVA revealed that this was not statistically significant. The second and third principal components accounted for 11% and 10% of this variability, respectively. One-way ANOVA showed that these PC scores were significant with respect to land class ($p<0.05$), and thus these scores were used to plot the distribution of average land-use class (Fig. 5.2a). Both the PC plot (Fig. 5.1a) and the corresponding loading graph (Fig. 5.2b) for land-use showed a high level of site dispersion on the PC plane.

One-way ANOVA showed that none of the first four PC scores significantly explained variability between soils communities associated with different textural classes.

Table 5.1 Soil location, physicochemical and community composition

Soil ID	Location	X coordinate	Y coordinate	Land use class	Texture	pH	%OM	PC1** (32%)	PC2** (11%)	PC3** (10%)	PC4** (8%)
JC1	Johnstown	302208	116139	Wood	Silt	5.3	8.7	-6.24	-3.67	0.5	-0.02
JC2	Johnstown	302810	116740	Grass	Clay	5.5	8.1	-1.46	-0.41	1.88	-0.98
JC3	Johnstown	301060	116213	Grass	Clay	6.4	17.6	-0.01	-0.64	2.12	-2.47
JC4	Johnstown	301135	116018	Grass	Sand	6.6	7.4	-2.79	-2.23	1.2	-2.07
KIL	Kilworth	183458	101902	Grass	Silt	6.2	8.1	2.14	-3.89	1.76	0.16
MP	Moorepark	183458	101902	Grass	Silt	6.0	8.4	3.74	-2.69	0.62	0.26
SOLO	Solohead	186147	139352	Grass	Clay	6.2	4.4	3.86	0.82	1.95	-1.4
OP1	Oakpark	273681	179296	Wood	Sand	4.8	12.0	1.35	2.15	0.26	0.07
OP2	Oakpark	273885	179117	Wood	Sand	7.3	7.0	-0.74	1.45	1.03	-0.45
OP3	Oakpark	273876	179631	Arable	Clay	6.6	5.9	2.76	3.35	-3.31	-0.59
OP4	Oakpark	273198	179662	Arable	Clay	6.4	6.5	-0.5	0.77	0.37	-0.1
OP5	Oakpark	273234	179298	Grass	Silt	5.9	5.8	3.37	-1.67	0.35	-0.83
OP6	Oakpark	273299	180080	Grass	Silt	7.5	7.5	4.61	0.32	-0.08	-2.11
OP7	Oakpark	272898	179883	Wood	Silt	4.5	20.9	4.66	1.22	-5.28	-2.86
OP8	Oakpark	272575	180235	Arable	Silt	6.1	6.1	4.01	0.01	-0.34	0.83
OP9	Oakpark	272421	179353	Arable	Sand	6.5	7.3	-0.77	0.25	-1.57	2.71
OP10	Oakpark	272659	179149	Arable	Sand	7.1	5.1	5.26	-0.91	-1.06	-0.06
OP11	Oakpark	272397	178842	Arable	Sand	5.5	10.2	-5.8	-0.01	-1.78	-0.71
OP12	Oakpark	272689	178977	Arable	Sand	5.9	12.5	1.83	0.36	-1.73	2.29
OP13	Oakpark	273296	179032	Arable	Sand	5.6	9.1	3.5	-0.9	-1.83	0.67
OP14	Oakpark	273296	179032	Arable	Silt	7.0	5.0	4.23	-1.53	-2.58	1.94
OP15	Oakpark	271310	179227	Arable	Silt	5.7	9.9	3.83	-0.05	-0.62	1.76
OP16	Oakpark	271311	180075	Arable	Sand	7.2	5.4	1.13	-2.13	-2.1	3.39
KD1	Kildalton	246069	123071	Grass	Silt	7.1	5.5	-1.89	-2.7	2.4	-0.94
KD2	Kildalton	246000	123000	Arable	Clay	6.8	7.5	-7.19	-2.08	-1.26	1.27
KD3	Kildalton	246552	123050	Arable	Clay	6.5	4.6	-2.97	0.9	0.94	1.55
KD4	Kildalton	246552	123050	Arable	Silt	5.6	4.8	-1.9	1.91	0.07	3.49
KD5	Kildalton	246552	123050	Wood	Sand	6.1	7.5	-3.69	1.48	-0.94	-1.24
KD6	Kildalton	246552	123050	Wood	Clay	5.9	4.7	-3.83	1.87	-0.34	-0.25
KD7	Kildalton	246552	123050	Wood	Sand	7.5	10.8	-3.09	2.35	-0.86	-2.07
KD8	Kildalton	246552	123050	Wood	Clay	6.5	5.2	-2.3	0.09	0.92	-0.17
KD9	Kildalton	246198	122738	Grass	Silt	6.9	8.5	2.46	-2.07	3.72	-0.42
KD10	Kildalton	246547	122570	Wood	Clay	5.2	4.7	-1.5	-1.01	2.05	1.79
KD11	Kildalton	247550	122831	Grass	Silt	7.9	4.4	-4.08	-3.02	1.03	-1.22
KD12	Kildalton	247098	122624	Grass	Silt	6.4	10.7	-0.52	-1.26	1.99	-0.3
KD13	Kildalton	246832	122427	Wood	Silt	7.1	6.4	-3.24	3.5	-0.82	-1.47
KD14	Kildalton	246832	122427	Arable	Sand	6.0	4.9	-1.76	3.55	0.46	-0.34
KD15	Kildalton	246800	122400	Arable	Clay	6.6	6.3	0.49	4.38	0.99	1.18
KD16	Kildalton	246800	122400	Arable	Clay	7.7	7.4	2.04	2.43	0.22	0.2

**=P<0.001

Table 5.2 Reference numbers used for PLFAs, shorthand and systematic names (c.f. Figs. 5.1 and 5.2)

PLFA ID, shorthand and systematic name					
ID	**Shorthand**	**Systematic name**	**I.D**	**Shorthand**	**Systematic name**
1	PLFA_1	unidentified	28	PLFA_17	unidentified
2	PLFA_2	unidentified	29	PLFA_18	unidentified
3	PLFA_3	unidentified	**30**	**i17:0**	**Me. 15-methylhexadecanoate**
4	**12:0**	**Me. dodecanoate**	31	PLFA_19	unidentified
5	PLFA_4	unidentified	32	PLFA_20	unidentified
6	**2-OH 12:0**	**Me. 2-hydroxydodecanoate**	**33**	**17:0c**	**Me. Cis-9,10-methylenehexadecaonate**
7	PLFA_5	unidentified	34	PLFA_21	unidentified
8	PLFA_6	unidentified	**35**	**17:0**	**Me heptadecanoate**
9	**14:0**	**Me.tetradecanoate**	36	PLFA_22	unidentified
10	PLFA_7	unidentified	37	PLFA_23	unidentified
11	PLFA_8	unidentified	38	PLFA_24	unidentified
12	**i15:0**	**Me. 13-methyltetradecanoate**	39	PLFA_25	unidentified
13	**a15:0**	**Me. 12-methyltetradecanoate**	**40**	**18:2w6c**	**Me. cis-9,12-octadecadienoate**
14	**15:0**	**Me. Pentadecanoate**	**41**	**18:1w9c**	**Me. cis-9-octadecanoate**
15	PLFA_9	unidentified	**42**	**18:1w9t**	**Me. trans-9-octadecanoate**
16	PLFA_10	unidentified	43	PLFA_26	unidentified
17	PLFA_11	unidentified	44	PLFA_27	unidentified
18	**3-OH 14:0**	**Me. 3-hydroxytetradecanoate**	**45**	**18:0**	**Me. octadecanoate**
19	**i16:0**	**Me. 14-methylpentadecanoate**	46	PLFA_28	unidentified
20	PLFA_12	unidentified	47	PLFA_29	unidentified
21	**16:1w7c**	**Me. Cis-9-hexadecanoate**	48	PLFA_30	unidentified
22	**16:1w7t**	**trans 9-hexadecanoic acid**	49	PLFA_31	unidentified
23	PLFA_13	unidentified	**50**	**19:0c**	**Me. cis-9,10-methyleneoctadecanoate**
24	**16:0**	**Me. hexadecanoate**	**51**	**19:0**	**Me. nonadecanoate**
25	PLFA_14	unidentified	52	PLFA_32	unidentified
26	PLFA_15	unidentified	53	PLFA_33	unidentified
27	PLFA_16	unidentified	**54**	**20:0**	**Me. eicosanoate**

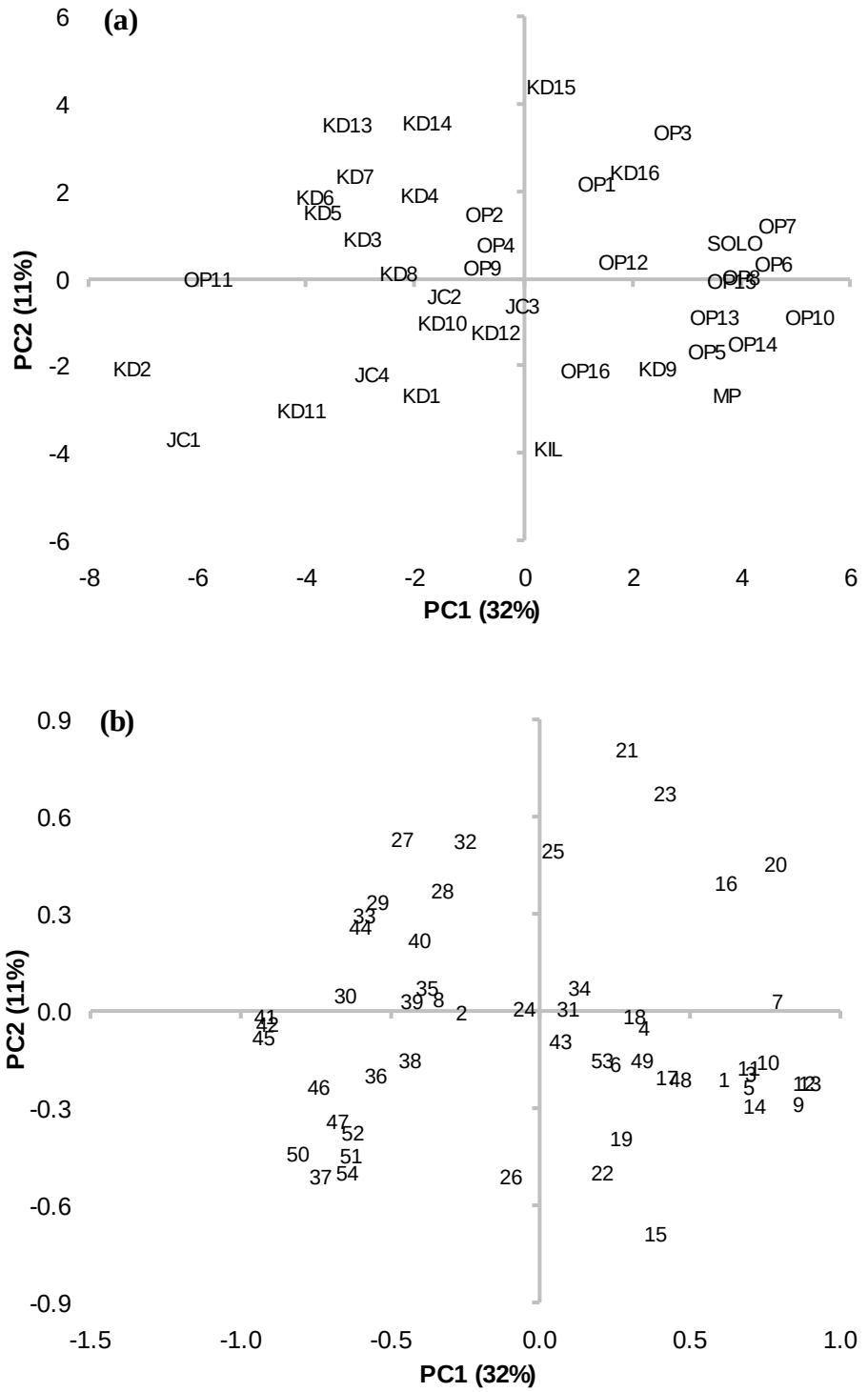


Fig. 5.1 (a) First and second principal components derived from average PLFA profiles of 39 soils according to soil ID and (b) loading plot showing PLFAs contributing to PC discrimination (n=3). See Table 5.2 for PLFA identification

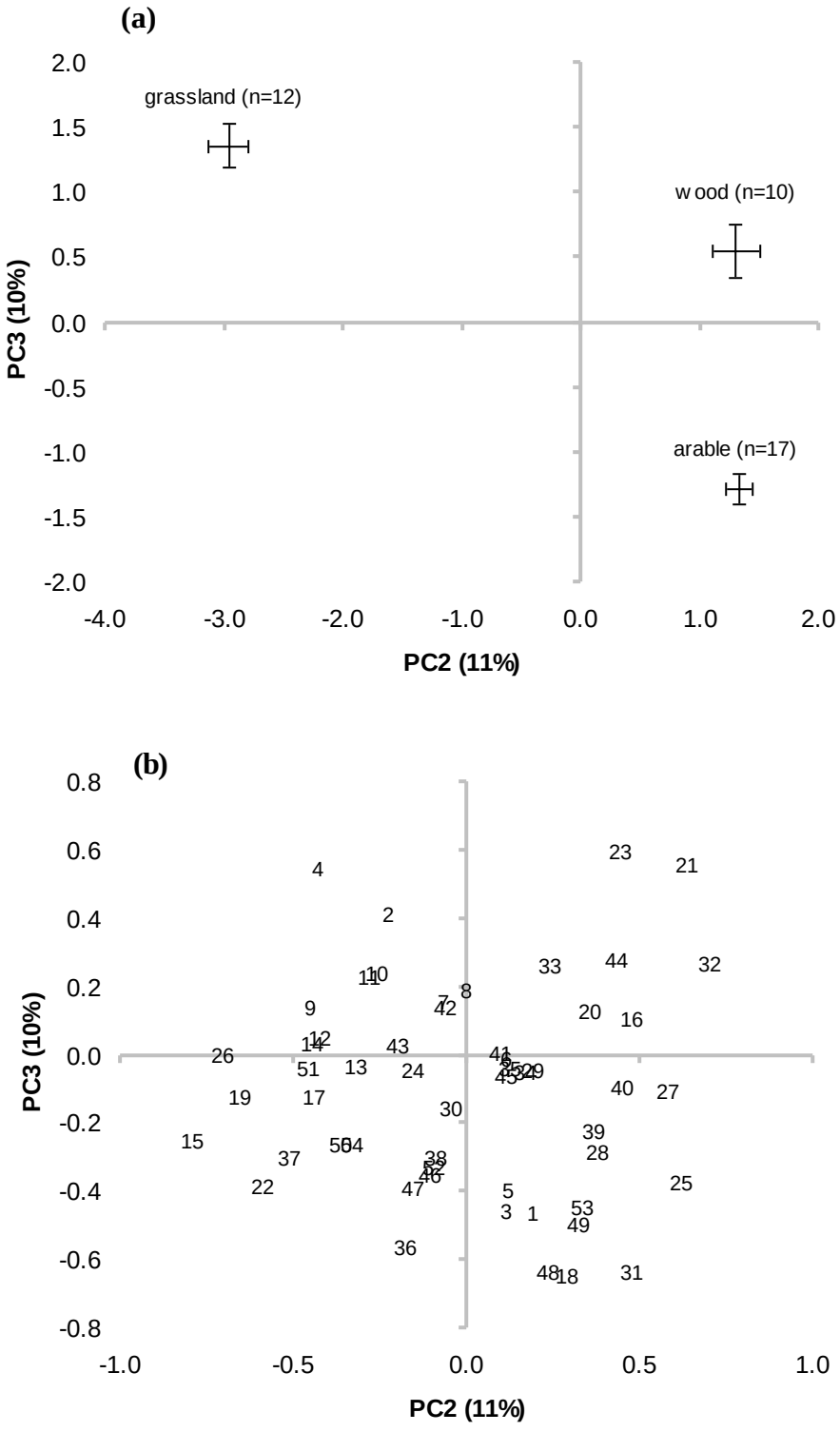


Fig. 5.2 Second and third principal components derived from average PLFA profiles of 39 soils according to (a) average land-use (error bars represent standard error, n shown in parenthesis) and (b) loading plot showing PLFAs contributing to PC discrimination. See Fig. 5.2 for PLFA identification

5.4. Discussion

The wide distribution of 39 soils on the PC plane indicated that the soils possessed contrasting communities, as was anticipated. PC analysis of average PLFA profiles for each land-use class revealed that the second and third components significantly explained 24% of the variation in community composition. This percentage is low, and the effect of land-use class on soil communities may have been confounded by treatments within each class. For example, the grassland class contained both organic and conventionally managed soil, the arable class contained both minimum and conventional tillage systems and different cereal crop species, and the wood class comprised forest and land used for willow and miscanthus production. Bossio et al., (1998) showed a significant difference in community composition between soil under organic and conventional management, associated with different carbon inputs. Feng et al., (2003) looked at differences in community composition in conventional and no-till cotton systems. This study showed significant differences in PLFA profiles between tillage practices during fallow and prior to cotton establishment. Cookson et al., (2008) investigated community composition according to a disturbance gradient associated with tillage. They showed that tillage largely influenced the soil physico-chemical environment, thus affecting the soil community. Molecular characterisation of bacterial communities associated with the rhizosphere of wheat, maize, rape and barrel clover were largely shaped by crop type, due to specific root exudate configuration (Haichar et al., 2008). Similarly, PC analysis of average PLFA profiles for each textural class showed that texture was not significantly associated with variation in community composition. Again, this could be due to low resolution of the hand texture method used to assign textural classes.

The aim of this screening process was to prescribe soils with different phenotypes for survival analysis. Thus we were interested in general land-use and textural composition to indicate where contrasting communities would be likely. Although this categorisation was crude, it was sufficient for this purpose, and the final suite of selected soils were more fully characterised to carry out a detailed multivariate analysis in order to delineate predominant influential factor involved in regulating pathogen decay.

The next step involved labelling these sites according to specific land-use and textural classes, pH and % OM, to visualise distribution of communities according to these site-specific parameters (Fig. 5.3). The aim of this process was to screen soils and pick an appropriate suite of 12 for further pathogen survival analysis. These 12 soils needed to represent a range of different combinations, to include different communities with similar physico-chemical composition and similar communities with different physico-chemical composition.

These particular physico-chemical traits were chosen as they have been reported to significantly influence microbial community structure in soil. Jangid et al., (2008) used molecular and PLFA community analysis to show significant differences between forest soil and agricultural soil, including pasture and cropland. A study by Steenwerth et al., (2002) obtained distinct PLFA signatures associated with 9 different land-use types on sand and sandy loam soil. These land-use types included irrigated and non-irrigated, annual and perennial grassland, with and without cultivation. Soil type and texture have been described as important determinants of community structure. For example, Girvan et al., (2003) used physiological and molecular techniques to show that bacterial communities clustered primarily according to soil type, despite distinctly different geographical locations and land-management practices. Additionally Bossio et al., (1998) reported that soil texture was more influential than season, management and spatial variation in determining community structure. However, the effect of texture was not observed here, likely due to broad textural classification. It has also been shown that soil pH could be used to predict the structure of the bacterial community, largely based on changes in proportions of acidobacteria, actinobacteria and bacteriodetes (Lauber et al., 2009). Also Fierer et al., (2006) showed that soil pH provided the best predictor of bacterial diversity and evenness based on ribosomal DNA fingerprinting, in a comprehensive study across North and South America. Frostegård et al., (1993) reported that an increase in soil pH was associated with an increase in Gram-negative and corresponding decrease in Gram-positive bacteria. Their work also showed that the liming process resulted in an increase in the proportion of actinomycetes. These studies demonstrate the importance of land-use, soil type, pH and OM in regulating soil community composition.

PC plots were used to investigate the distribution of soils according to community composition, and were labelled according to soil ID, land-use, texture, pH and % OM (Fig. 5.3a-e, respectively). First and second PC scores were deemed appropriate for plotting as they accounted for 43% of total variability. By comparing these graphs side by side, it was possible to visualise community differences with respect to physico-chemical properties. Circled data-points represent the final 12 soils selected for survival analysis. For example, soils KD2 and KD15 have different communities, yet have similar land-use, texture and physico-chemical properties. Soils KIL and KD15 represent different land-uses and have different communities, but again, texture and physico-chemical properties are similar. Soils OP1 and OP7 have similar communities. These were both collected from the wood land-use class and have similarly low pH levels. However they contrast in terms of texture and % OM. Soils JC3 and KIL have reasonably similar communities, on heavy grassland soil. They also have similar pH, but very different % OM. This selection process maximised the range of communities and physico-chemical properties to reflect all possible combinations.

The corresponding PLFA loadings were also plotted to determine the fatty acids responsible for variability between the 39 soils (Fig 5.1b). The wide distribution of PLFAs on the PC plane suggests that subtle shifts in the total suite of PLFAs detected were responsible for driving differences in community structure.

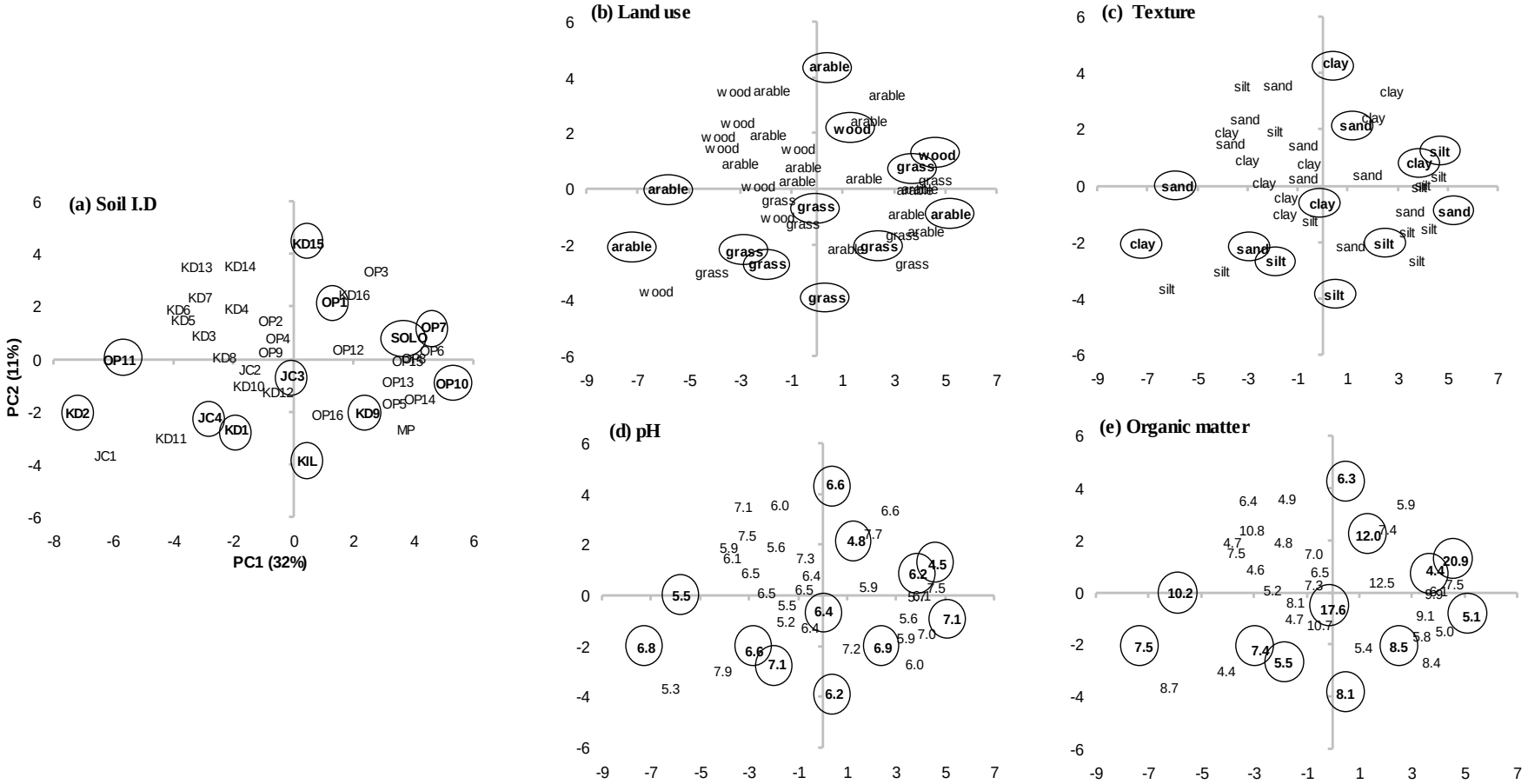


Fig. 5.3 First and second principal components derived from PLFA profiles of 39 soils according to (a) soil ID, (b) land-use, (c) texture, (d) pH and (e) % OM (n=3). Circled data-points represent final 12 soils selected for pathogen survival analysis

5.5. Conclusions

The aim of this experiment was to prescribe biologically and physico-chemically contrasting soils for subsequent pathogen survival analysis. It was expected that soils from different land-uses would provide the range of properties required, and this was investigated using a novel PC screening approach. It was shown that the soils possessed different edaphic and community compositions, such that an appropriate suite of 12 contrasting soils were selected to determine the main factors influencing pathogen decay, described in Chapter 6.

Chapter 6. Influence of soil communities derived from different land-uses on model pathogen survival

6.1. Introduction

Thus far, this work has investigated pathogen survival in the context of very different community composition, i.e. presence/absence of an intact microbial community (Chapter 3), and subtly different community composition (Chapter 4), i.e. variations on a native community developed in the same basic soil. Both instances were achieved by experimental manipulation. In order to investigate this pathogen:community interaction in a context more pertinent to the field situation, in this chapter we consider soils which were collected from different land-uses to obtain 'naturally' contrasting microbial communities. These soils were profiled to characterise differences in both biotic and physico-chemical composition (Chapter 5), and were inoculated with model enteropathogens to determine the principal factors influencing the decay of such pathogens.

In Chapter 5 it was shown that soils from different land uses contained specific and contrasting microbial community configurations. The aim of this experiment was to extend this knowledge further, and investigate the role of these communities in pathogen suppression. To date, pathogen survival in these sorts of contexts has been mostly investigated in relation to prevailing abiotic conditions, and the role of biology and interactions with the soil community has been generally overlooked. Abiotic factors known to affect pathogen survival include moisture, temperature, texture, pH, CEC, UV irradiation, OM and soil nutrient status (reviewed in van Elsas et al., (2011)). There is also some evidence to suggest an inverse relationship between pathogen survival and microbial diversity (van Elsas et al., 2007; Ibekwe et al., 2010). However, in Chapter 4 it was shown that the community phenotype is apparently more influential in regulating pathogen decay as compared to the inherent diversity. Some work has been carried out to demonstrate the effects of land-use and management on pathogen suppression. However, the pathogen survival response has been found to be variable and difficult to predict within a framework of complex interactions between site-specific factors, including current and historical land-use, the physico-chemical environment,

predominant management strategies, and resultant impact on community composition. A study by van Elsas et al., (2002) showed significant differences in community composition between bulk soil and rhizosphere soil of maize and oats. Additionally, suppression of the plant pathogen *Rhizoctonia solani* AG3 was greater in rhizosphere soil in this context, particularly in the case of maize. These observations were attributed to differences in soil microbial diversity. In contrast Williams et al., (2007b) showed no effect of a maize rhizosphere on survival of *E. coli* O157 as compared to bulk soil when manure was applied. This study focused mainly on the influence of rhizodeposits and nutrient status on *E. coli* survival, and did not take community composition into account. Other work has shown distinct differences in *E. coli* O157 survivability between manure-amended sterile and non-sterile soil, partly attributed to antagonism from the soil microbial community (Jiang et al., 2002). However, other parameters were also implicated in regulating *E. coli* decay, including soil temperature and the soil:manure ratio (Jiang et al., 2002). Franz et al., (2005) showed that when manure spiked with *E. coli* O157 was applied to soil, inactivation was more rapid in soils under organic as compared to conventional management. The rate of *E. coli* decline was positively associated with total N, nitrate and total carbon. It was suggested that nutrient availability may have stimulated an increase in the native community, resulting in increased competition with *E. coli*. This differential inactivation between management strategies was not observed for *S.* Typhimurium, signalling that this organism may be more resistant to such competitive effects. However, this work focused on the influence of nutrients on survival, and as the community was not measured, provided only circumstantial evidence of pathogen-specific biological interactions associated with different management strategies. Further work by Franz et al., (2008b) showed that in organic soil amended with manure, variation in *E. coli* O157 survivability could be best explained by levels of dissolved organic carbon. However, a significant correlation was also found with microbial community diversity.

It is therefore unclear whether edaphic or biotic factors play a dominant role in governing pathogen survival, particularly as few studies have considered both in a coherent manner. The aim of this experiment was then to investigate the survival of different pathogen types in relation to naturally-contrasting community phenotypes associated with different land-uses. It was hypothesised that soil biology, specifically

the configuration of the microbial community, would be more influential in regulating pathogen decay than soil physico-chemical composition

6.2. Materials and methods

6.2.1. Soil preparation and microcosm establishment

The 12 soils prescribed for this experiment, as described in Chapter 5, namely JC3, JC4, KIL, SOLO, OP1, OP7, OP10, OP11, KD1, KD2, KD9 and KD15, were homogenised and sieved to 4 mm, and stored in loosely tied plastic bags at 4^{o}C until use. The WHC for each soil was determined by the method described in Franz et al., (2011). Moisture content was then adjusted to achieve similar friability between soils by wetting up or drying on the bench as appropriate. Following adjustment, soil moisture was measured by oven-drying at 105^{o}C for 24 hours, and expressed as a percentage of WHC. Pathogen survival microcosms were established by weighing out aliquots of 5 g soil into sterile 40 ml polypropylene tubes. The tubes were covered with Parafilm to prevent moisture loss during incubation. Caps were then loosely replaced to allow for gas exchange, whilst minimising the risk of contamination. All tubes were stored at 10^{o}C until inoculated with the pathogen suspension. This temperature was selected as it reflects the average annual topsoil soil (0-10 cm) temperature in Ireland.

6.2.2. Soil physico-chemical characterisation

The soils that were selected for subsequent pathogen analysis were also characterised for a wider range of physico-chemical parameters than were adopted for Chapter 5. For this purpose, these soils were oven dried at 40^{o}C and mechanically sieved to 2 mm. Physico-chemical analyses was carried out at Brookside Laboratories Inc., New Knoxville, Ohio, for total exchange capacity, pH, % OM, phosphorus (Mehlich III; Bray II; Olsen); exchangeable cations (Ca, Mg, K, Na); extractable minor cations (B, Fe, Mn, Cu, Zn, Al); % organic C; % C; % N, C/N ratio; % clay; % silt and % sand in triplicate. Details of these analyses are summarised in Table 6.1 below.

Table 6.1 Physico-chemical tests used to characterise soils

Soil test	Brief description	Reference
Total exchange capacity	TEC by summation	Ross, 1995
pH	1:1 H_20	McLean, 1982
Organic matter	Loss on ignition at 360^oC	Schulte and Hopkins, 1996
Olsen P		Olsen and Sommers, 1982
Bray II P		Bray and Kurtz, 1945
Mehlich III P		Mehlich, 1984
Extractable ions		Mehlich, 1984
Inorganic nitrogen	1 M KCl cadmium reduction	Dahnke, 1990
Total carbon and nitrogen	Combustion	McGeehan and Naylor et al. (1988); Nelson and Sommers, 1996
Soil texture	Hydrometer	ASTM D422, 2000

6.2.3. PLFA analysis

It was necessary to stagger the determination of the survival of the four pathogens for reasons of practicality associated with workload. Prior to inoculation all soil samples were stored at 10^oC. However, it was considered plausible that during the 4-week storage period, the microbiological community configuration might change. Therefore the precise community configuration to which each pathogen was exposed was determined by undertaking PLFA analysis at the outset of each inoculation. Samples were prepared for PLFA analysis immediately prior to pathogen inoculation, as described in Section 2.4.

6.2.4. Pathogen inoculation and survival analysis

Pathogen inoculum cultures were prepared as described in Section 2.1.3. Microcosms, consisting 5 g soil in sterile polypropylene vials (40 ml), were individually inoculated with approximately 10^8 cells of either *L. monocytogenes*, *S.* Dublin, *E. coli* O157 or environmentally persistent *E. coli* (Lys 9), which constituted 10^7 cells g^{-1} soil. Final soil moisture following inoculation, at which soils were incubated, was then expressed as a percentage of WHC (Table 5.1). Pathogen inoculation was staggered into pathogen-specific batches involving all twelve soils simultaneously. For each pathogen batch, a pool of 96 microcosms per soil type were inoculated at three instances selected at random after 2 hours (denoted T_0) and 2, 4, 8, 16, 32, 64 and 110 days (denoted T_2, T_4, T_8, T_{16}, T_{32}, T_{64}, T_{110}). Soils continued to be incubated at 10^oC throughout these

experimental periods. *L. monocytogenes* could not be quantified at T_{110}, due to excessive growth of background microflora on Oxford agar plates. Therefore, survival data for this organism are only presented to T_{64}. Enumeration was carried out as described in Section 2.1.4.

6.2.5. Data analysis

Pathogen survival data were collected by counting characteristic colonies on pathogen-specific agar. Triplicate counts for each soil treatment were averaged and were plotted as CFU g^{-1} (dry weight). These data were used to fit exponential decay curves and calculate the average death rate for each pathogen within the context of each soil treatment, as described in Section 2.5.3. PLFA profiles were analysed by PCA as described in Section 2.5.4, and mean scores determined for each soil independently for each batch. PC analysis was also applied to the entire data set across the four batches, and the mean scores determined accordingly. Replicate physico-chemical data provided by Brookside Laboratories Inc. was averaged to indicate the composition of each soil. Finally, physico-chemical, community and k-values were averaged per soil, and entered into a forward stepwise regression model (SAS, Version 9.1) to investigate the predominant influential factor involved in pathogen decay regulation across the 12 soil types.

6.3. Results

6.3.1. Soil properties

To reiterate, the final 12 soils comprised 6 grassland, 4 arable and 2 wood land-use classes. A wide range of values were manifest for most edaphic properties determined (Table 6.1). For example, OM ranged over 10%, Olsen P an order of magnitude, and C/N ratio two-fold (Table 6.1). The pH range was quite extensive, spanning 2 orders of magnitude. Biomass C ranged over 614 µg C g^{-1} soil (dry weight) (Table 6.1).

6.3.2. PLFA analysis

Soils were labelled alphabetically (Table 6.2) and PLFAs were labelled numerically (Table 6.3) to aid visualisation during PC analysis. When PLFA profiles were analysed collectively across all four batches, there was a highly significant effect of batch ($P<0.001$) and soil ($P<0.001$), but no significant interaction between these terms ($P=0.2$-0.5) for any of PC1-4, which accounted for 66% of the variability between soils. Ordination of mean scores for each batch showed significant separation of all four circumstances, with Batch 3 being notably separated by PC1, 2 and 4 (Fig. 6.1a). Batches 1, 2 and 4 tended to cluster in the ordinations but were nonetheless significantly separated by PCs 1-3 (Fig. 6.1c).The combinations of individual PLFAs contributing to these PCs were generally dispersed and there was no dominant effect of any particular PLFA (Fig. 6.1b and 6.1d). Ordination of PC1 and PC2 for the PLFA profiles associated with each soil independently showed concomitantly wide dispersion (Fig. 6.2), with notable differences between the ordinations in the four circumstances (Fig. 6.3). Again, ordination of corresponding PLFA loadings in this case showed that neither PC1 nor PC2 was dominated by particular PLFA types (Fig. 6.4).

6.3.3. Pathogen decay rates – curve fitting

Pathogens declined in an exponential manner in all instances (Fig. 6.5). There was visual evidence to suggest different survival characteristics between soils, particularly in the case of *L. monocytogenes* (Fig. 6.5b), in the form of notably different curve gradients. This was confirmed by differences in pathogen-specific death rates (Table 6.4). Overall, there was an order of magnitude difference between highest and lowest death rates, observed for *E. coli* Lys 9 in JC4 and *L. monocytogenes* in OP7, respectively. The exponential decay function was a significant fit ($p<0.05$) for all pathogens within each soil.

There was an association between larger positive PC values for PC1 and death rate for *S.* Dublin (Fig. 6.6a) and *E. coli* Lys 9 (Fig. 6.6b), which was not apparent for the other two pathogens. The highest death rate for *E. coli* O157 was in the KIL soil, which was associated with a significantly greater score for PC1 (Fig. 6.6d). There was no association between death rate and PC1 for *L. monocytogenes* (Fig. 6.6b). There was a

strong linear relationship between death rate and PC2 for *S*. Dublin (Fig 6.7a, $P<0.01$) and *L. monocytogenes* (Fig. 6.7b, $P<0.01$), where higher death rates were associated with smaller negative values in PC2. There was no association between death rate and PC2 for either *E. coli* Lys 9 (Fig. 6.7c) or *E. coli* O157 (Fig. 6.7d). There was no association between death rate and PC score for PC3 and PC4 (Appendix 3D and 3E).

Table 6.2 Soil edaphic and biomass properties

Soil ID*	Soil	Land use category	Specific land use	Total cation exchange capacity (ME 100 g^{-1})	pH	% Organic matter	Olsen P (ppm)	% Organic C	% C	% N	C:N ratio	% Clay	% Silt	% Sand	Moisture (% field capacity)**	Biomass C (µg C g^{-1} dry soil)
A	JC4	grass	grazing	11.7	6.2	6.1	46.7	3.3	3.8	0.4	10.4	13.6	34.1	52.3	46.3	356
B	JC3	grass	grazing	13.2	5.6	6.4	54.7	3.6	3.8	0.4	10.6	12.9	31.6	55.5	48.3	384
C	OP7	wood	forestry	26.4	7.5	5.2	8.0	4.1	4.6	0.3	18.0	25.2	33.0	41.8	41.1	230
D	OP1	wood	willow	17.8	7.6	5.4	94.7	3.5	3.8	0.3	12.1	21.0	22.0	57.0	38.8	263
E	KD2	arable	maize	10.0	6.4	3.0	70.3	1.5	1.9	0.2	8.8	19.4	32.6	48.1	43.0	81
F	KD15	arable	cabbage	10.0	7.0	3.8	47.0	2.0	2.5	0.2	11.0	21.9	35.9	42.1	44.3	113
G	KIL	grass	grazing	14.8	5.5	7.2	138.0	3.9	5.8	0.4	13.4	16.5	32.3	51.2	41.7	381
H	KD11	grass	grazing	11.8	5.7	6.8	54.7	3.9	4.2	0.4	10.2	19.3	45.8	43.9	45.0	485
I	SOLO	grass	grazing	22.0	6.4	13.8	240.0	5.9	7.6	0.8	9.8	23.5	41.4	35.1	54.5	695
J	OP10	arable	till + mustard cover	13.3	6.9	4.2	178.7	3.0	3.0	0.3	11.2	10.7	22.4	66.9	35.6	122
K	KD9	grass	grazing	12.9	5.9	7.8	86.7	4.0	4.6	0.5	9.6	20.3	36.2	43.6	44.7	415
L	OP11	arable	till, sprayed	12.9	6.9	4.2	145.0	2.8	2.8	0.3	10.3	8.8	21.7	69.5	36.0	115

*c.f. Figs. 6.2, 6.3, 6.6 and 6.7

**Moisture content at which samples were incubated

Table 6.3 Reference numbers used for PLFAs, shorthand and systematic names (c.f. Figs. 6.1 and 6.4)

PLFA ID, shorthand and systematic name					
ID	**Shorthand**	**Systematic name**	**ID**	**Shorthand**	**Systematic name**
1	**12:00**	**Me. dodecanoate**	**28**	**i17:0**	**Me. 15-methylhexadecanoate**
2	PLFA_1	unidentified	29	PLFA_18	unidentified
3	**14:00**	**Me.tetradecanoate**	30	PLFA_19	unidentified
4	PLFA_2	unidentified	**31**	**17:0c**	**Me. Cis-9,10-methylenehexadecaonate**
5	PLFA_3	unidentified	32	PLFA_20	unidentified
6	PLFA_4	unidentified	**33**	**17:00**	**Me heptadecanoate**
7	**i15:0**	**Me. 13-methyltetradecanoate**	34	PLFA_21	unidentified
8	**a15:0**	**Me. 12-methyltetradecanoate**	35	PLFA_22	unidentified
9	**15:00**	**Me. Pentadecanoate**	36	PLFA_23	unidentified
10	PLFA_5	unidentified	**37**	**18:2w6c**	**Me. cis-9,12-octadecadienoate**
11	**2-OH 14:0**	**Me. 2-hydroxytetradecanoate**	**38**	**18:1w9c**	**Me. cis-9-octadecanoate**
12	PLFA_6	unidentified	**39**	**18:1w9t**	**Me. trans-9-octadecanoate**
13	**3-OH 14:0**	**Me. 3-hydroxytetradecanoate**	40	PLFA_24	unidentified
14	PLFA_7	unidentified	41	PLFA_25	unidentified
15	**i16:0**	**Me. 14-methylpentadecanoate**	**42**	**18:00**	**Me. octadecanoate**
16	PLFA_8	unidentified	43	PLFA_26	unidentified
17	**16:1w7c**	**Me. Cis-9-hexadecanoate**	44	PLFA_27	unidentified
18	PLFA_9	unidentified	45	PLFA_28	unidentified
19	PLFA_10	unidentified	46	PLFA_29	unidentified
20	**16:00**	**Me. hexadecanoate**	47	PLFA_30	unidentified
21	PLFA_11	unidentified	**48**	**19:0c**	**Me. cis-9,10-methyleneoctadecanoate**
22	PLFA_12	unidentified	**49**	**19:00**	**Me. nonadecanoate**
23	PLFA_13	unidentified	50	PLFA_31	unidentified
24	PLFA_14	unidentified	51	PLFA_32	unidentified
25	PLFA_15	unidentified	52	PLFA_33	unidentified
26	PLFA_16	unidentified	**53**	**20:00**	**Me. eicosanoate**
27	PLFA_17	unidentified			

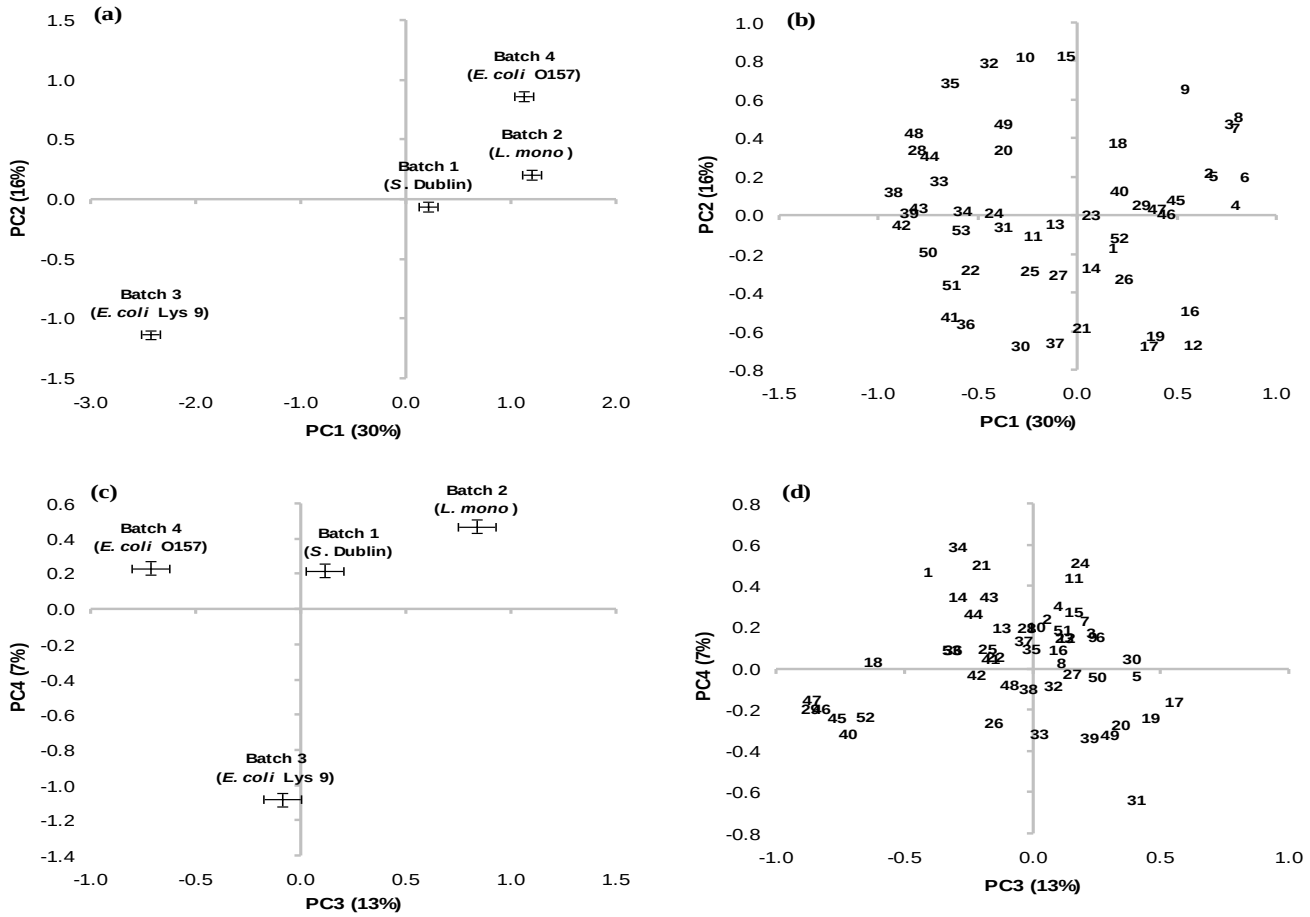


Fig. 6.1 Ordinations of (a) first and second principal components derived from average PLFA profiles according to pathogen batch ± standard error (n=36), (b) corresponding PLFA loadings, (c) third and fourth principal components derived from average PLFA profiles according to pathogen batch± standard error (n=36) and (d) corresponding PLFA loadings. See Table 6.2 for PLFA identification

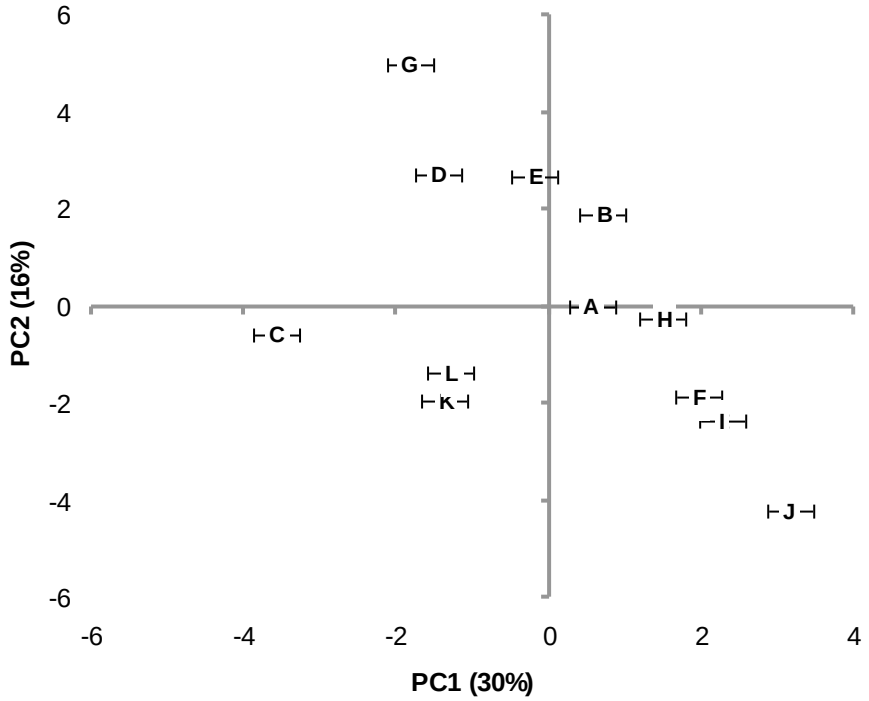


Fig. 6.2 First and second principal components derived from average PLFA profiles with respect to soils. Data represent PC scores ± standard error (n=12). See Table 6.1 for soil identification

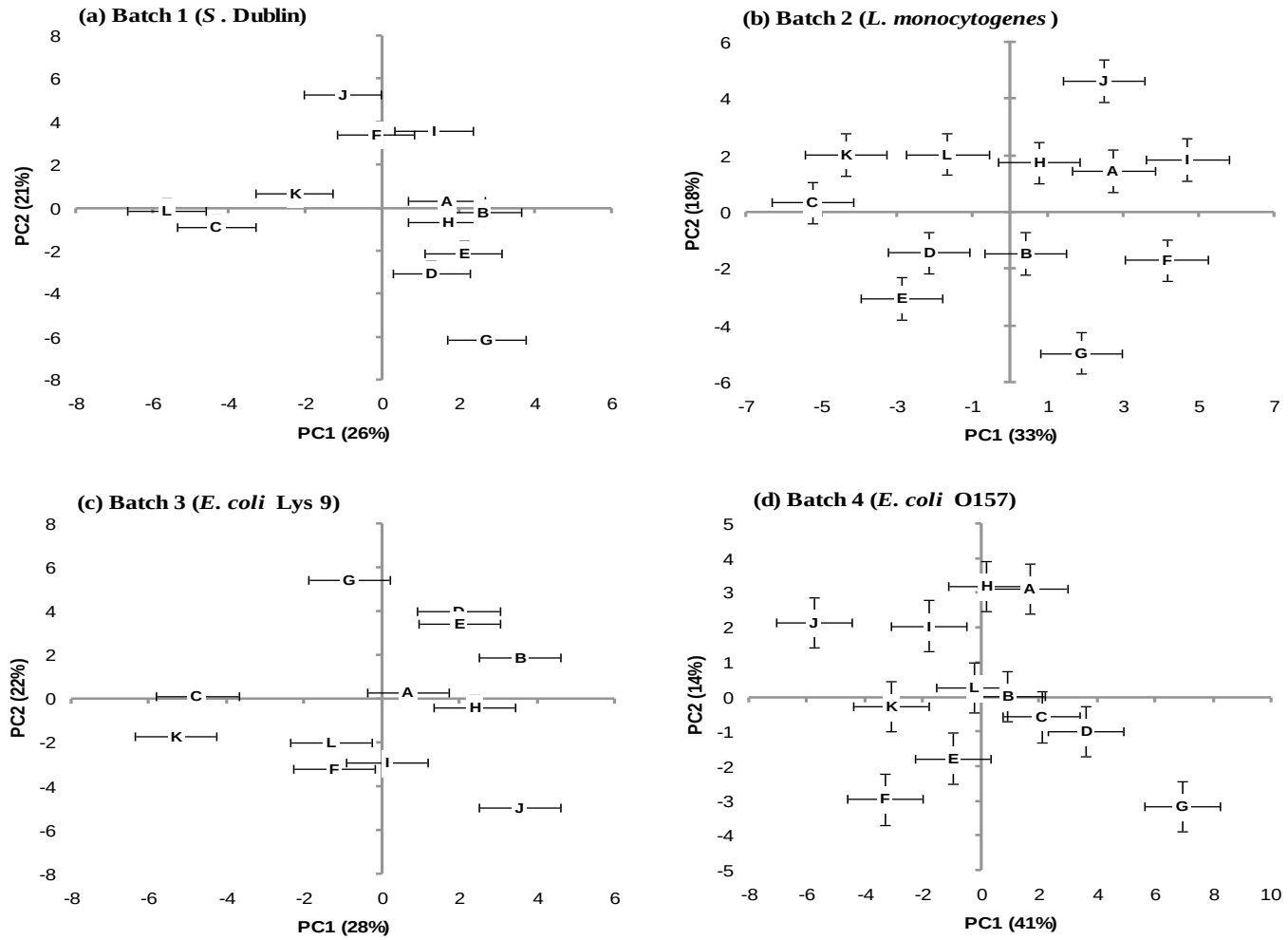


Fig. 6.3 Ordination of soils according to first and second principal components derived from individual PLFA profiles for each pathogen batch at respective T_0's for (a) *S.* Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157Data represent PC scores ± standard error (n=3). See Table 6.1 for soil identification

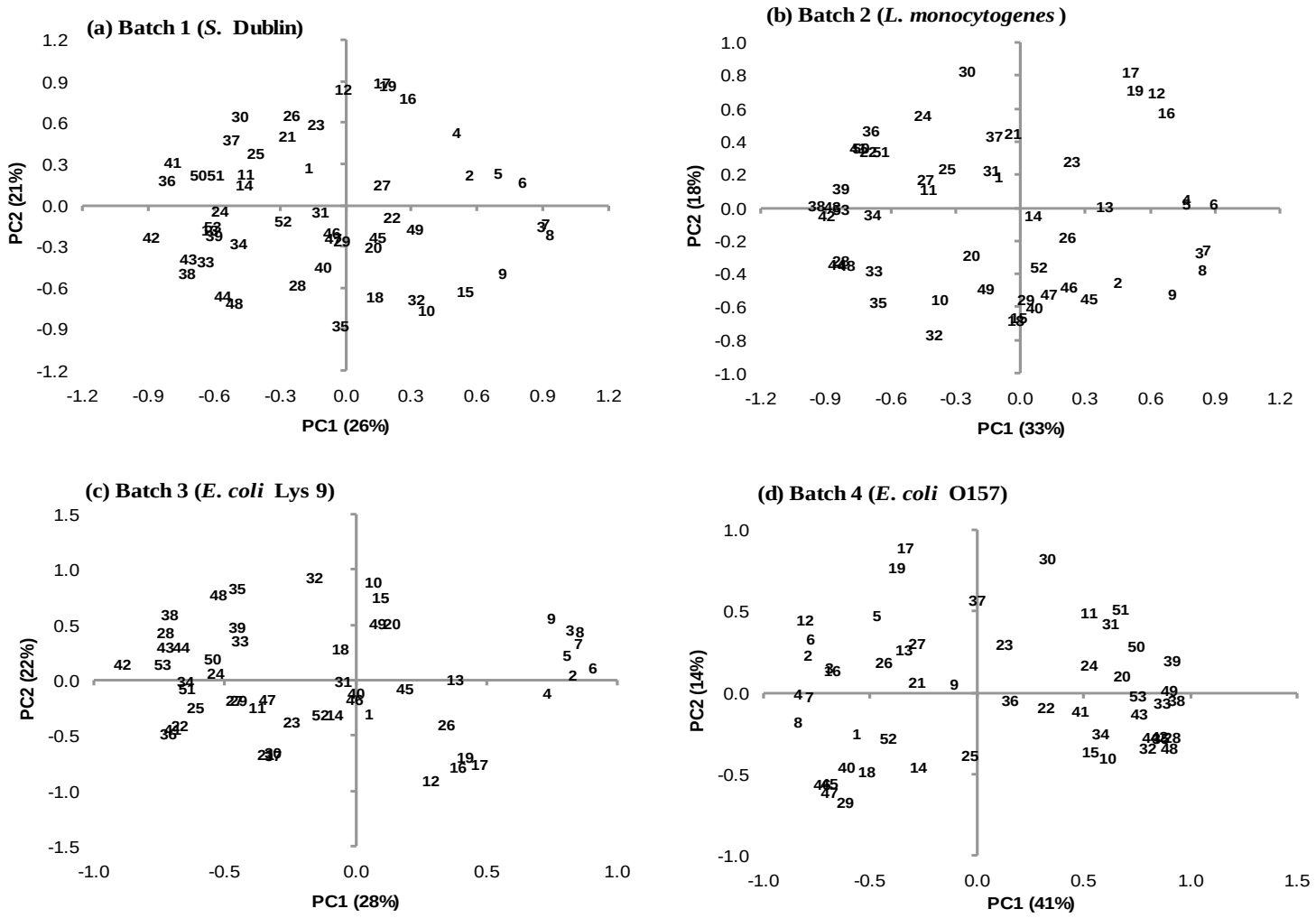
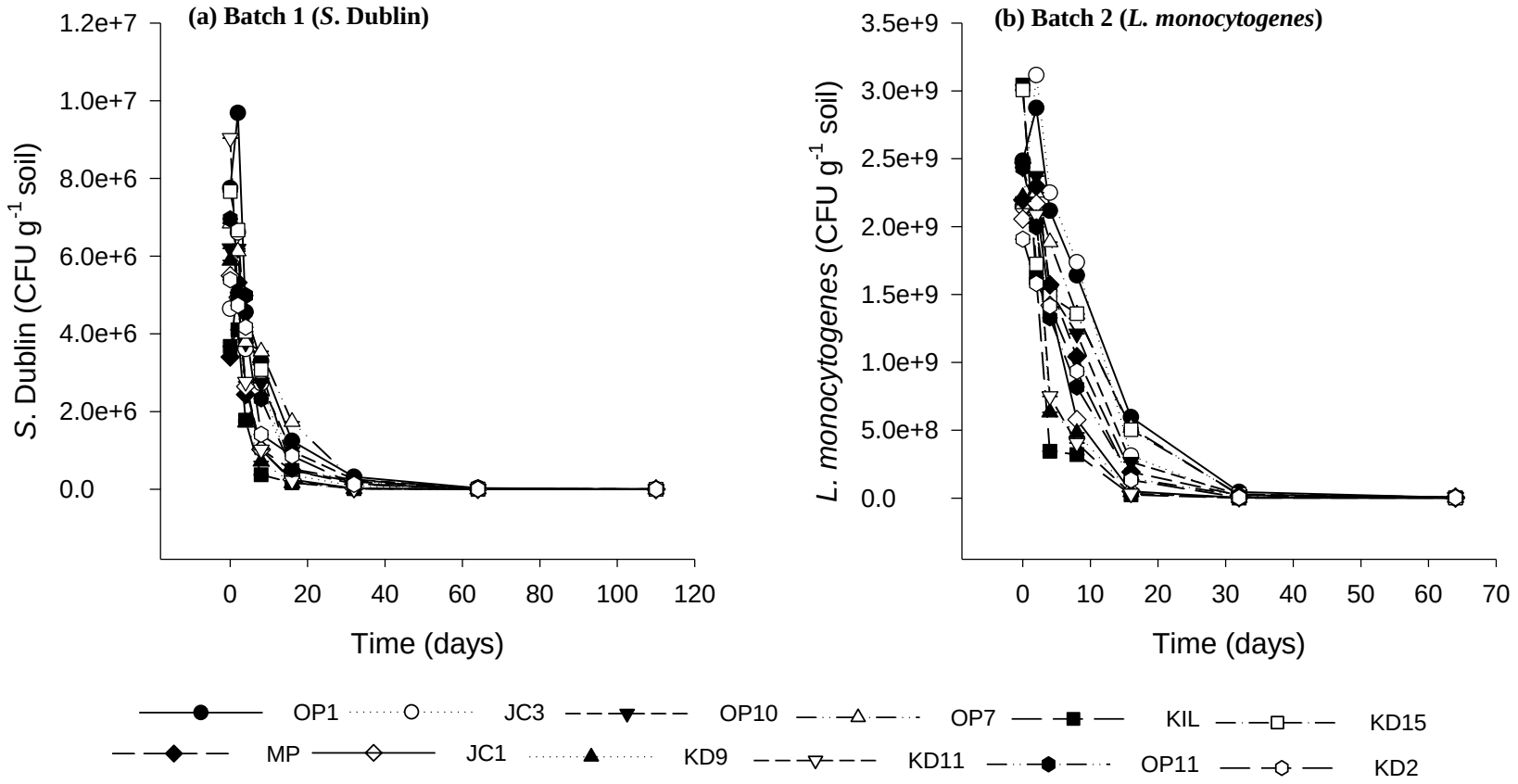


Fig. 6.4 Loading plots associated with first and second principal components derived from individual PLFA profiles for each pathogen batch at respective T_0's for (a) *S.* Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. Data represent PC loadings (n=3). See Table 6.2 for PLFA identification


(a) Batch 1 (S. Dublin)
S. Dublin (CFU g-1 soil)
1.2e+7
1.0e+7
8.0e+6
6.0e+6
4.0e+6
2.0e+6
0.0
0
20
40
60
80
100
120
Time (days)
(b) Batch 2 (L. monocytogenes)
L. monocytogenes (CFU g-1 soil)
3.5e+9
3.0e+9
2.5e+9
2.0e+9
1.5e+9
1.0e+9
5.0e+8
0.0
0
10
20
30
40
50
60
70
Time (days)
OP1
JC3
OP10
OP7
KIL
KD15
MP
JC1
KD9
KD11
OP11
KD2

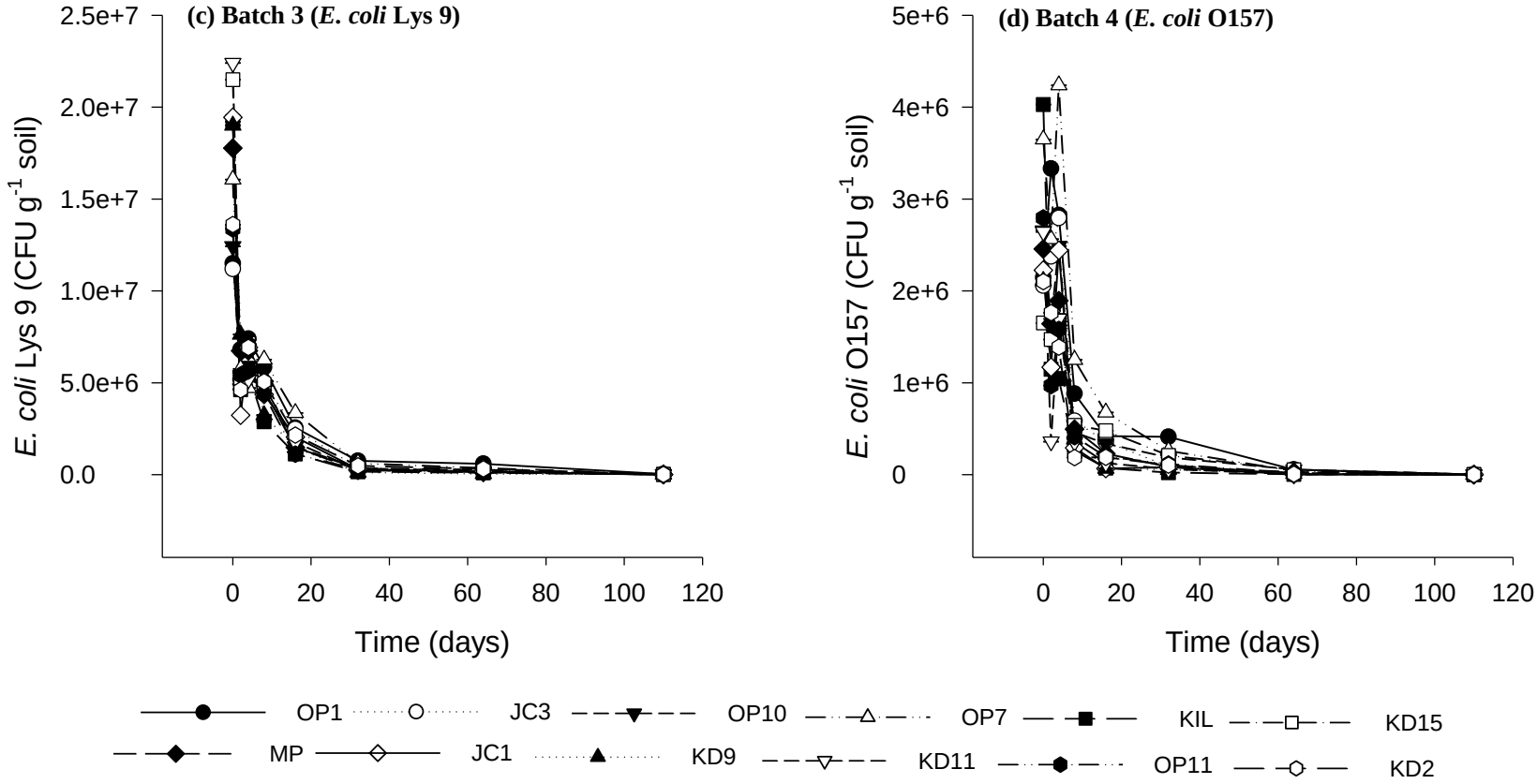


Fig. 6.5 Decay curves for (a) *S*. Dublin, (b) *L. monocytogenes*; (c) *E. coli* Lys 9 and (d) *E. coli* O157 following inoculation to soil microcosms. Data represent average CFU g^{-1} soil (dry weight) ± standard error (n=3) Note that error bars fall within confines of treatment symbols

Table 6.4 Decay rates of pathogens introduced into soils from different land-uses

	K-values (days^{-1})*			
Soil ID	***S.* Dublin**	***L. monocytogenes***	***E. coli* LYS 9**	***E. coli* O157**
JC4	0.22±0.02	0.13±0.03	0.89±0.29	0.13±0.05
JC3	0.10±0.03	0.07±0.02	0.11±0.02	0.09±0.03
OP7	0.09±0.01	0.07±0.02	0.42±0.11	0.09±0.03
OP1	0.12±0.01	0.07±0.02	0.08±0.01	0.09±0.03
KD2	0.12±0.03	0.10±0.02	0.17±0.04	0.17±0.03
KD15	0.13±0.03	0.12±0.02	0.55±0.11	0.09±0.03
KIL	0.24±0.05	0.40±0.08	0.59±0.12	0.51±0.07
KD11	0.25±0.03	0.22±0.05	0.71±0.15	0.22±0.06
SOLO	0.13±0.04	0.10±0.02	0.28±0.05	0.14±0.03
OP10	0.13±0.01	0.10±0.01	0.19±0.04	0.13±0.04
KD9	0.23±0.03	0.21±0.06	0.30±0.05	0.16±0.02
OP11	0.13±0.02	0.14±0.01	0.22±0.04	0.24±0.06

*Exponential decay model significantly fit curves for all pathogens and treatments ($P<0.05$)

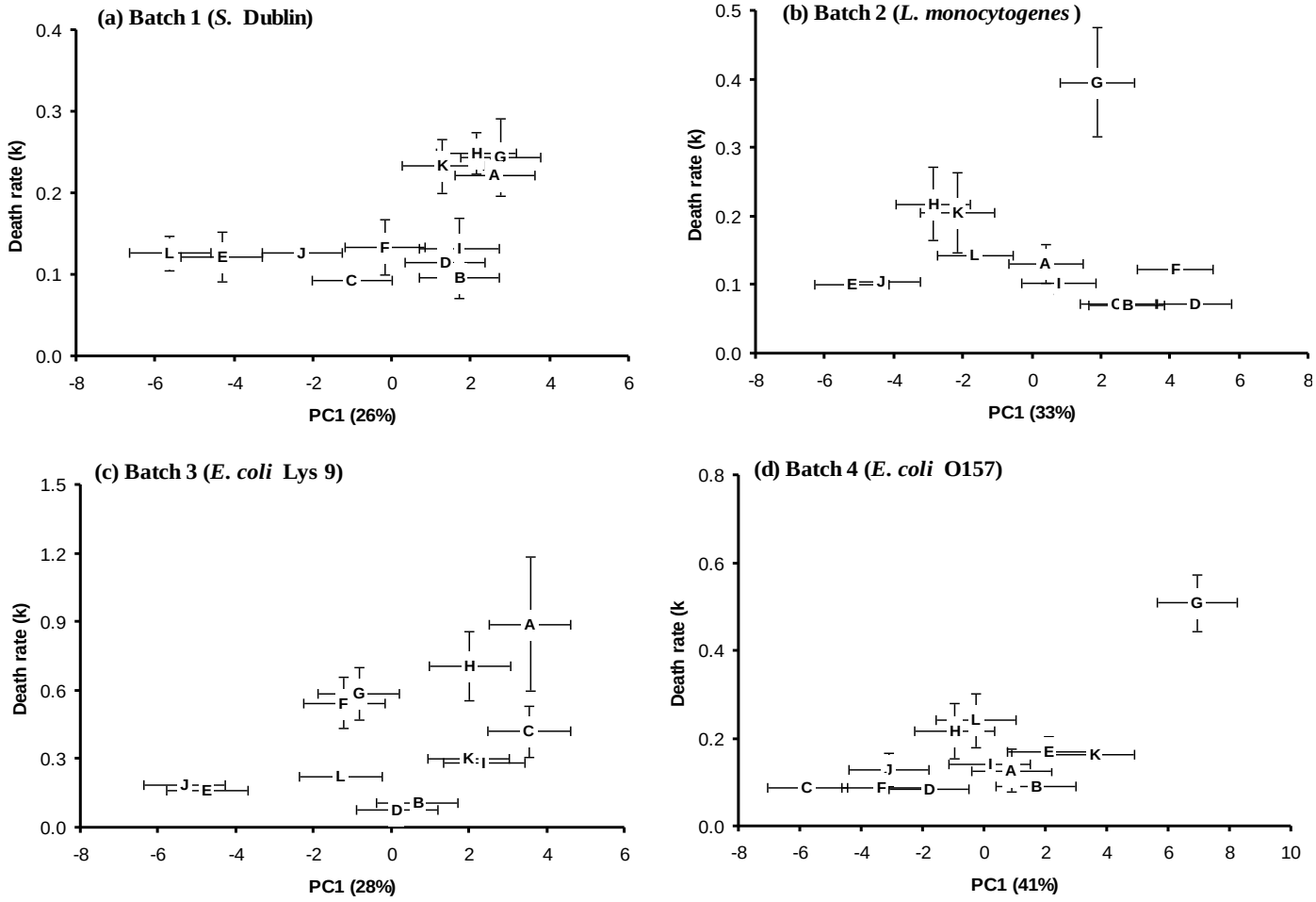


Fig. 6.6 Relationship between death rate of (a) *S.* Dublin, (b) *L. monocytogenes,* (c) *E. coli* Lys 9 and (d) *E. coli* O157 and community structure represented by first principal component derived from average PLFA profiles associated with each batch. Data represent average values ± standard error (n=3). See Table 6.1 for soil identification

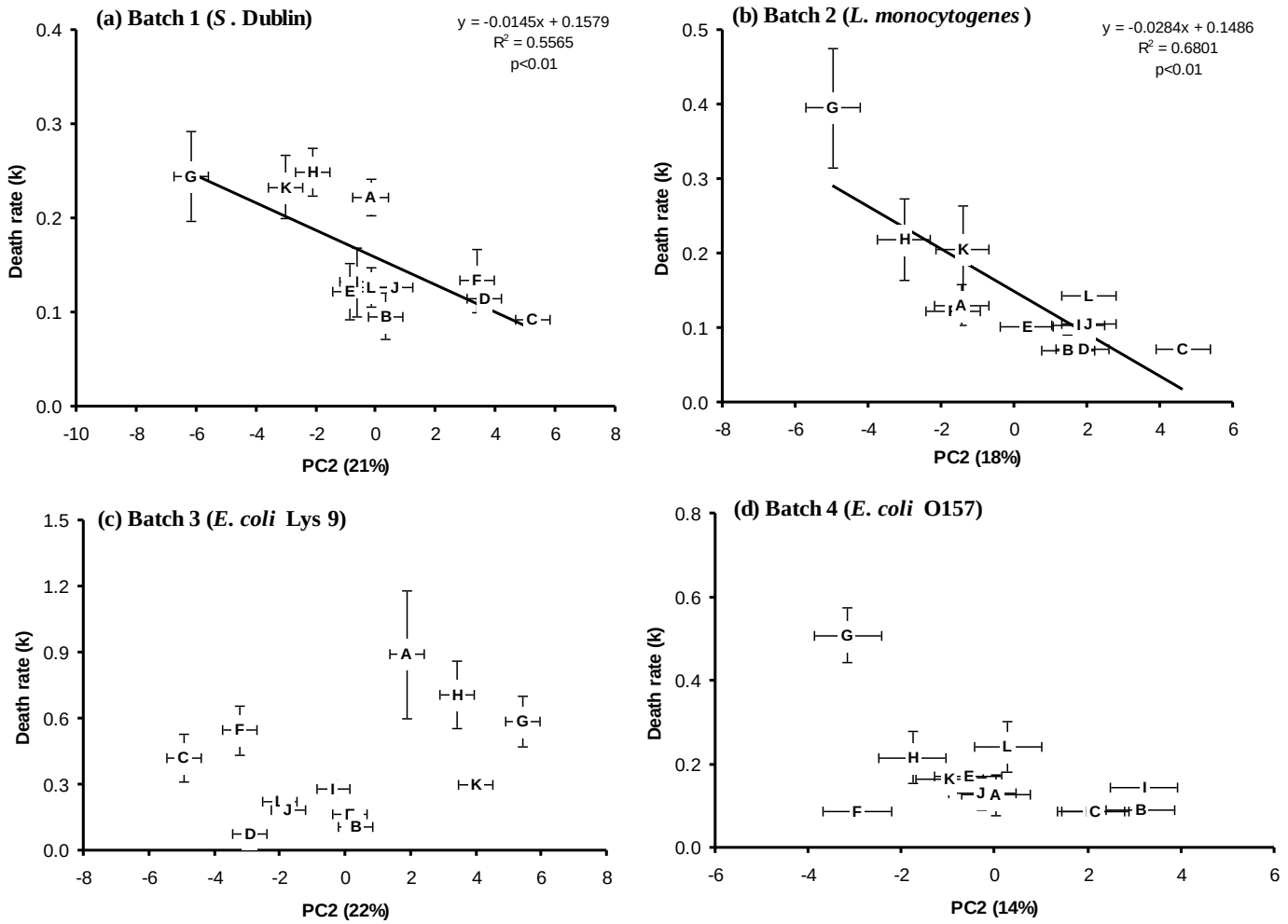


Fig. 6.7 Relationship between death rate of (a) S. Dublin, (b) *L. monocytogenes,* (c) *E. coli* Lys 9 and (d) *E. coli* O157 and community structure represented by second principal component derived from average PLFA profiles associated with each batch. Data represent average values ± standard error (n=3). See Table 6.1 for soil identification

6.4. Discussion

Pathogens were inoculated into a suite of soils with a view to assessing survival capacity. Pathogen inoculation took place on a weekly basis in a series of pathogen-specific batches. PC analysis of average PLFA profiles showed significant differences in community composition between batches (Fig. 6.1). This indicates community composition within soils was not entirely conserved during the inoculation period, even at 10^{o}C, as anticipated. This effectively means that the respective pathogens were inoculated into different community contexts. Whilst it was shown in Chapter 3 that incubation at 4^{o}C curtails biological interactions effectively, at least in terms of pathogen suppression, to have used this temperature in this series of experiments would have necessitated a further staggered pre-incubation phase, which in itself would not guarantee congruence across all batches. In particular, communities associated with *E. coli* Lys 9 were apparently different in comparison to those associated with remaining pathogens. There are a couple of potential reasons for this phenomenon. Soil community shifts over storage time has been previously reported (e.g. Petersen and Klug, 1994; Wu et al., 2009). If this differential development was coherently associated with time, one would expect the fourth (final) inoculation event to be more isolated on the PC plane. However, communities associated with the final inoculation are similar to those associated with first and second inoculation events. Alternatively, community differences may have been associated with variations in the extraction procedure. Samples were processed according to batches corresponding to the pathogen inoculation sequence; therefore it is possible that PLFA extraction efficiencies varied between batches.

PC analysis also showed highly significant differences in community phenotypic composition between soils, as anticipated (Fig. 6.2). Dispersal of soils within the PC trait space indicates that a wide variety of community contexts were included in this study. The lack of a significant soil by batch interaction is evidence that the *relative* differences between communities were conserved over time when all batches were considered together. Therefore, it is likely that differences in absolute community composition between batches

were associated with a combination of factors including sample storage, preparation and PLFA extraction efficiency.

Thus, pathogen response to the community context across the four batches in this study cannot be compared in robust statistical terms. However, the fact that relative differences in community composition were conserved provides tentative evidence that communities were broadly congruent between batches; consequently similarities in behaviour can be tentatively evaluated.

The PC plots associated with each batch, representative of the range of soil communities present at respective pathogen-specific T_0's (Fig. 6.3, Table 3A) depict the precise community contexts to which the pathogens were exposed. PC analysis revealed significant differences between soil communities within each batch. However, differences in the distribution of soil communities within the PC trait space provides further evidence of variation in absolute community composition over time. The PLFA loadings associated with these principal components showed that differences in a range of PLFAs contributed to the significant discrimination between communities associated with these 12 soils (Fig. 6.4). Therefore, discrimination between communities associated with different land uses was based on shifts in the total microbial cohort in this case. This contrasts with other work that has looked at the effect of different treatments on community configurations in soil. For example, Bossio et al., (1998) found associations between fatty acid signatures and organic, low input and conventional management, suggesting that particular groups were responsible for variation between management regimes. Similarly Frostegård et al., (1997) showed distinct differences in PLFAs associated with manure and those associated with soil, when investigating the impact of manure hotspots on microbial community dynamics. The lack of dominant PLFAs in this case may be due to comparison of a variety of soil communities, encompassing many different land use treatments and soil types.

Different pathogen survival curves and death rates were manifest for each soil. Death rate tended to be greatest within the grass land-use class, and poorest in arable and wood land-use classes for all pathogens. Regression analysis showed that PC scores representative of community composition provided the best predictor of pathogen survival for 3 of 4 pathogens investigated (Fig. 6.6 and 6.7, $p<0.01$). However, the response of one

of these pathogens was skewed by an outlier that was associated with the community within the KIL soil (Fig. 6.6d) and thus this correlation is not presented. There was no significant correlation between survival and any of the other physicochemical factors tested – such terms were clearly excluded from the multiple regression procedure.

Therefore communities associated with soils from the grassland land-use class, in particular KIL, were more suppressive towards the pathogens than those associated with soils from arable or wood land-use classes. PLFA loadings show that the suppressive effect observed in this case was of a general rather than specialist nature, caused by interactions with the total microbial consortium within these soils, rather than with specific microbial groups. Differential survival between grassland and arable soils has been shown previously in the context of the plant pathogen *Rhizoctonia solani* AG3. Molecular analysis revealed greater microbial diversity in grassland as compared to arable soils, resulting in an enhanced suppressive effect and reduced spread of pathogenic fungal hyphae (van Elsas et al., 2002). It is possible that grassland represents intermediate disturbance levels, as compared to higher disturbance associated with arable and lower disturbance associated with woodland soils. Intermediate disturbance tends to promote diversification of the microbial community (Jangid et al., 2008), which could potentially account for greater suppression witnessed in grassland here. This diversification may have been more pronounced for KIL, as this soil community was particularly antagonistic towards the introduced pathogens.

It was shown that pathogen survival was predominantly affected by the soil microbial community. Other work has found circumstantial evidence that antagonistic interactions with the soil community can regulate pathogen decline. For example, Jiang et al., (2002) compared survival of *E. coli* O157 in manure-amended autoclaved and unautoclaved soil, and noted rapid inactivation in unautoclaved soil. This response was attributed to soil microbiota and was contingent on other factors including temperature and manure:soil ratio. Similarly, *Salmonella enterica* serovar Newport showed greater initial population increase, slower rate of decline and longer survival periods in manure-amended sterile as compared to non-sterile soil. Again, this response was partially attributed to microbial antagonism (You et al., 2006). Further, work by Franz et al., (2008b) investigated

the main biotic and abiotic factors influencing persistence of *E. coli* O157 in a suite of manure-amended soils. This work showed that in the presence of manure, pathogen survival was highly correlated with levels of dissolved organic carbon. In organic soils, a secondary correlation was identified with microbial diversity described by molecular techniques. These results suggested that pathogen survival times were mostly contingent on nutrient supply, and could be reduced by amending soil with high quality manure containing a comparatively lower and more complex nutrient load, in order to minimise nutrient availability to opportunistic pathogens. However, the soils that were used in our experiment did not receive any nutrient addition during the incubation period. Potentially, the role of soil biology in pathogen suppression becomes more apparent in the absence of nutrient input.

In this instance, none of the physico-chemical factors could explain differences in pathogen survival between soils, when PC scores representing the community context were included in regression analysis. In the mesocosm experiment in Chapter 4, the survival characteristics of *S.* Dublin and *E. coli* Lys 9 were significantly correlated with 'subtle' differences community composition within **experimentally-manipulated soils**, whereas those of *L. monocytogenes* and *E. coli* O157 were not. In comparison, the survival of all pathogens except *E. coli* Lys 9 was significantly correlated with contrasting and unrelated communities associated with the **natural soils** used in this experiment. However, the correlation for *E. coli* O157 in PC1 is questionable, due to the idiosyncratic effect of the KIL soil. This was not reported as significant in the context of Fig. 6.7d. Nonetheless, this organism still showed evidence of a response to the community context, as high death rates were manifest in soil communities represented by high PC scores.

E. coli Lys 9 did not respond to the community context in this experiment. This organism responded to subtle community differences that manifest within the same soil type in Chapter 4, one would expect this organism to be more susceptible on exposure to more the obvious community differences achieved here. However the total lack of correlation for *E. coli* Lys 9 could be attributed to the matter that the community context to which this organism was exposed differed to that of other pathogens, due to differential community development during the incubation period. Had the community context for *E.*

coli Lys 9 been congruent to that of the others, one might hypothesise that a similar significant correlation between survival of this organism and community composition may have been observed. Alternatively, *E. coli* Lys 9 may have responded differently as it is an environmentally persistent isolate, and has been shown to form naturalised populations and persist in soil for a number of years (Brennan et al., 2010a). Therefore it may not be as susceptible to community interactions as other organisms used in this study. However as community composition differed between inoculation events, this is merely speculation. Future work should focus on survival of these pathogens in different community contexts, where these differences are conserved between inoculations. In doing this, pathogen survival characteristics could be effectively compared and unusual survival behaviour of *E. coli* Lys 9 could be further investigated. Addition of nutrients should also be considered, as pathogens are typically introduced to soil in an organic carrier material such as manure or sewage sludge.

6.5. Conclusions

It was hypothesised that contrasting soil community phenotypes derived from different land-uses would play an important role in regulating pathogen survival, irrespective of differences in physico-chemical composition. This was strongly supported for 2 out of 4 pathogens tested, with some support in the case of a third pathogen. This provides further evidence of the importance of the community context in pathogen suppression, also demonstrated in Chapter 4. These findings highlight the need to include some measure of community composition in predictive models, to get an accurate estimation of pathogen decay potential in soil.

Chapter 7. Main findings, general discussion and future research

7.1. Main findings

The aim of this project was to investigate the relationship between microbial community structure and pathogen survival in soil. This was considered to be an appropriate research topic, as previous work on pathogen survival has focused primarily on the role of abiotic factors. Some limited work on the role of soil biology has been carried out but this has been directed toward diversity, and comparatively little is known regarding the effect of the community context. Diversity is often considered at a genetic level, and is then based on the literal number of different species present in a community (i.e. 'richness'), whereas structure is more concerned with the absolute and relative proportions of such species. The phenotype represents the literal manifestation of the gene:environment interaction, and can be considered both at an individual and community scale. It was hypothesised that pathogen survival would be negatively correlated with diversity. This is because complex communities contain many different species that are capable of utilising a wide variety of nutrient sources. Therefore many of the functional and physical niches would be occupied, thus rendering it difficult for the pathogen to persist. It is also arguably likely that more complex soil communities would contain a relatively greater proportion of antagonists and predators, that could interact with the pathogen and thus in general cause pathogen decline. In contrast, communities that are simple in nature contain comparatively fewer species; thus niches may be under-exploited and the likelihood of interacting with antagonists and predators is also reduced. This environment may be conducive to pathogen survival. This over-arching hypothesis was investigated in the circumstances of three distinctly different community contexts. Firstly, we looked at pathogen response to biological extremes, where the soil community was either intact or eliminated by sterilisation. This clearly demonstrated that soil biology had a significant impact on pathogen survival. Secondly, soils were experimentally manipulated to create a nominal gradient of community complexity and sequence of community structure. This process resulted in subtle differences in community composition between soils, which were sufficient to cause

significant differences in pathogen survival rates in some instances. Thirdly, pathogen survival was investigated in the context of a wide range of natural communities derived from field soils. This showed significantly different survival profiles between soils from different land-uses, predominantly attributed to the associated community phenotype. All three experiments clearly show evidence of significant interactions between soil biology and pathogen decay. The main findings of this work are highlighted, and these are discussed in further detail below.

1. The decay rate of *E. coli* O157 was inversely related to the presence of native community, and this entirely curtailed at 4°C.
2. The soil physico-chemical environment directed the development of inoculated communities
3. There was some evidence of an inverse relationship between community complexity and pathogen decay rates
4. The soil community context was more influential in regulating pathogen survival than the inherent diversity
5. Soils from different land-uses possessed different community phenotypes
6. Pathogen death rates in soils from different land-uses were significantly affected by the soil community; no other abiotic factor tested could effectively further explain variation in pathogen decay between soils
7. Pathogen survival behaviour was associated with a combination of a range of PLFAs, indicating suppression is caused by interactions with the entire community rather than with single biotic components within the community, i.e. context-dependent indicating a general suppressive effect
8. Different pathogens displayed unique survival characteristics when exposed to different community contexts

7.2. Effect of different community contexts on pathogen survival

This section summarises key experimental outcomes, limitations and contribution to knowledge. It also highlights the common thread between experiments and how they were designed to collectively explore the impact of community structure on pathogen survival.

7.2.1. Intact community

It was hypothesised that the survival of *E. coli* O157 would be negatively affected by the presence of an intact microbial community (Chapter 3). *E. coli* survival was stable at 4^{o}C irrespective of soil biology, attributed to low metabolic activity. However, growth was observed in sterile soil at 18^{o}C. This was attributed to availability of unoccupied niche space, functional and physical, generated by the sterilisation process. Conversely, the population declined in non-sterile soil at 18^{o}C, due to negative interactions with the native community. Therefore the hypothesis was supported. There was evidence to suggest that growth and survival characteristics differed between sandy loam and clay soils, however these differences were not statistically significant. It was speculated that differential survival between soil types may have been due to differences in community composition. However, this experiment was designed to investigate the impact of the overarching presence or absence of a native community on survival, rather than the impact of community composition. Thus, the phenotypes within these soils were not characterised, and communities could not be compared.

Nonetheless, these results have important implications for slurry spreading in cool temperate climates such as Ireland. The incubation temperature regime was chosen to approximate soil temperatures in early spring when slurry spreading takes place. Slurry-derived pathogens may enter the soil when temperatures are low and form a reservoir that may increase when temperatures rise. The extent of this increase may be partly regulated by the presence of an intact microbial community. In this case, low temperatures suppressed *E. coli*, but also suppressed kill-off by the native community. *E. coli* decline only occurred at 18^{o}C when the native community was metabolically active. This provides evidence that suppression was based on biological rather than physico-chemical interactions.

This led to the question of how community composition, specifically complexity, could limit the establishment of introduced pathogens.

7.2.2. Gradient of community complexity

An experiment was designed to manipulate microbial diversity and community complexity, to investigate the relationship with survival of common slurry pathogens including *E. coli*, *Salmonella* and *Listeria* spp. (Chapter 4). This was carried out using a soil dilution approach, which was based on the assumption that rare microbial groups would be diluted out in low dilutions, while more common groups would be conserved in high dilution. This logic was successfully applied previously to assess the relationship between diversity and function. It was hypothesised that there would be an inverse relationship between pathogen survival and diversity, due to competitive pressure, lack of niche space and negative interactions associated with increasing community complexity.

Phenotypic analysis showed statistically significant differences in community composition between resultant dilutions. This was caused by a combination of fatty acids, suggesting that differences within the total microbial cohort were responsible for discrimination between dilution treatments. However, the principal components technique used to analyse PLFA profiles is by definition very sensitive, and showed that only the first and third principal components could significantly account for 35% of variability between dilutions. Also dilutions were primarily separated by the third principal component. Taken together, this suggests that while phenotypic profiles were significantly different, the PLFA shifts associated with these differences were subtle. Therefore despite extensive dilution only slight modification of the eventual phenotypes were achieved. This may be associated with predominance of the soil environment in determining the resultant phenotype, such that communities converged irrespective of the composition of the original inoculum. This supports Griffiths et al., (2008), who found that when different communities were inoculated into the same soil, the resultant phenotypes were similar. Therefore we propose that interactions between biology, soil structure and physico-chemical composition shaped the phenotype within dilution treatments in this case. It would have been informative to

track the temporal development of the community, both phenotypically and genetically. These soils had similar phenotypes; however the genotype, which is species-specific and offers more resolution than the phenotype, may have been very different between dilutions. The phenotype was here chosen as the preferred method of community characterisation as it represents the actual manifestation of the gene:environment interaction, at the community scale (Kibblewhite et al., 2008). It is therefore the biotic construct with which the pathogens interacted when they entered the soil environment in these studies. However, it does not provide the same resolution as the genotype at species level, and thus species diversity indices cannot be accurately derived from phenotypic data (Frostegård et al., 2010). In this case, a diversity effect was assumed, as it was a logical consequence of the dilution-manipulation approach adopted, and previous studies have confirmed this to be the case (e.g. Griffiths et al 2004, Crawford et al 2011). However, a detailed genetic characterisation in addition to phenotypic analysis may have provided a more comprehensive description of the community in this case. Nonetheless, intended treatment levels with different community configurations were apparent at the end of the incubation period, and therefore it was possible to test the hypothesis for this experiment in a statistically-robust manner.

Pathogen decay differed between soil dilution treatments. There was evidence of a diversity effect for two of the pathogens tested, reflected by the weak negative relationship between death rate and extent of dilution. This has been shown previously, where *E. coli* survival was enhanced by increasing soil fumigation levels (van Elsas et al., 2007; Ibekwe et al., 2010). However, this work has shown that pathogen death rates were in fact more sensitive to changes in the community phenotype. This is an important finding as it suggests that the community context plays a more important role than the inherent diversity in regulating pathogen survival. Thus survival is less related to the number of different species or microbial groups present, but rather to what species or groups are present, and the shape that they take, i.e. the community fingerprint. For instance, two native communities could possess equal diversity, but different phenotypic configurations. Therefore, these communities will interact differently with the pathogen invader, such that the pathogen may persist in one community, yet decline in the other. Equally, it may also be

possible for two communities to have different diversity based on species composition, but the same phenotype, regulated by soil:environment interactions (Fig. 7.1).

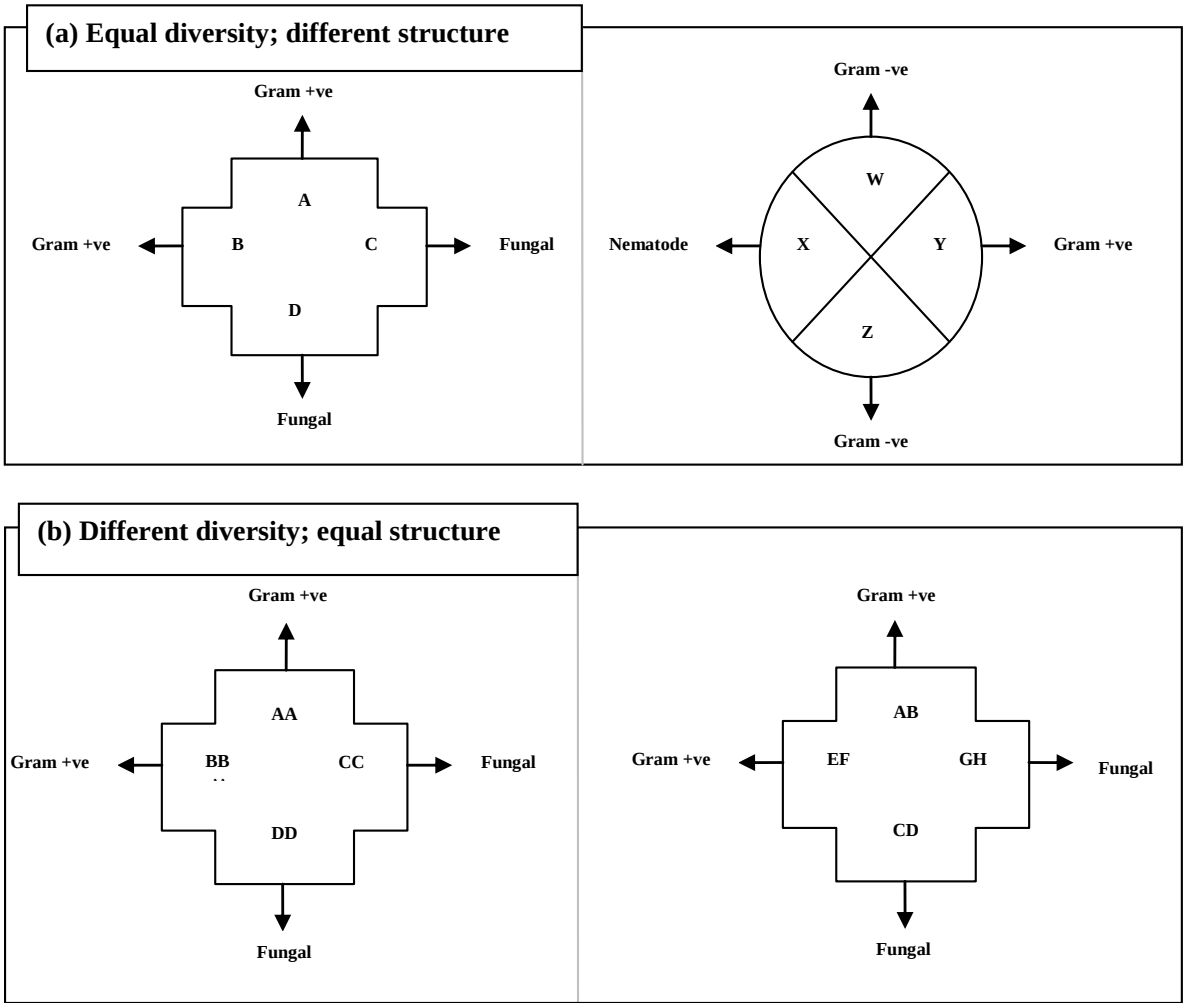


Fig. 7.1 Microbial communities with (a) equal diversity and different phenotypic structure and (b) different diversity and equal phenotypic structure. Species notated alphabetically, arrows point to phenotypic groups

The model pathogens tested displayed different survival characteristics. This is also of note, as the limited research that has been carried out on the effects of soil biology to date has focused primarily on the response of *E. coli* as a model organism (van Elsas et al., 2007; Ibekwe et al., 2010; Liang et al., 2011). However, these results suggest that survival may be specific to pathogen type, and influenced by factors such as cell structure, physiology, origin and ability to compete for resources within the confines of limited niche space. Therefore, the behaviour of *E. coli* cannot necessarily be extended to predict the behaviour

of other organisms in soil, and this highlights the need to include a range of different model pathogens in future studies.

In this case, there was some support for the original hypothesis, stating an inverse relationship between microbial diversity and pathogen survival. However, survival was apparently more influenced by community structure rather than diversity. Particular community phenotypes may have established with decreasing complexity, such that the pathogen could persist. This could potentially account for the limited diversity effect, and again points toward the importance of the phenotypic community context. These results raised the question of how pathogens would respond when exposed to randomly distributed soil communities derived from different land-uses, and whether the community would remain influential, irrespective of differences in the soil physico-chemical environment.

7.2.3. Natural communities

As expected on the basis of previous work, e.g. Jangid et al., (2008); Lauber et al., (2008), different land-uses supported significantly different community phenotypes (Chapter 5). Similar to Chapter 4, discrimination between soil treatments was caused by a range of PLFA forms. A screening approach based on principal component analysis was then used to select an appropriate range of soils, to assess the effect of the phenotype on pathogen survival (Chapter 6). It was hypothesised that soil community composition would be more important in regulating pathogen survival than other physico-chemical factors.

Different death rates were observed between soil treatments. There was some evidence to suggest that pathogen decay was more rapid within the grassland land-use soils as compared to arable or wood land-use classes. Multiple regression analysis showed that death rates were dominantly and significantly associated with community structure reflected by PC scores for three out of the four pathogens studied; addition of abiotic parameters into the regression model had no significant effect upon accounting for the variance in the fit. This significant correlation was questionable for *E. coli* O157, yet there was still evidence of an association between high death rates and particular community phenotypes. Therefore, the hypothesis was supported. Overall, this suggests that grassland

was associated with greater decay of the pathogens. However, land-uses in this case were broadly classified, and encompassed additional confounding treatments, with respect to plant species and management regime. In addition, there may have also been residual legacy effects of previous land-use on the soil community, depending on the length of time in cultivation. Therefore, the observation that greater pathogen decay was associated with the phenotype in grass *per se* is speculative and further work is required to tease out the complexities of linking pathogen survival specifically to communities associated with land-use.

In this experiment, survival of an environmentally-persistent strain of *E. coli* (Lys 9) did not apparently respond to the encountered phenotype. However, *E. coli* Lys 9 was in fact inoculated into a demonstrably different community context as compared to other model organisms, due to community shifts that occurred during the incubation period. This was unfortunate as consequently, pathogen survival behaviour was not then directly comparable in this experiment. However, as *E. coli* Lys 9 responded to subtleties in the community phenotype in Chapter 4, it was hypothesised that a significant interaction would have occurred, had it been exposed to the same phenotype as other pathogens in this case. Alternatively, it may have behaved differently, irrespective of the phenotype, as unlike the other pathogens it is a proven environmentally-persistent isolate, which is capable of long-term survival in Irish soils (Brennan et al., 2010a). To elucidate this, further work would be required to compare pathogen behaviour in a range of communities, to include different pathogens inoculated into the same communities at the same time. However, logistically this would be quite demanding and difficult to realise. Therefore in reality, it may be required to reduce either the number of soil communities or pathogens, to allow simultaneous inoculation into congruent soil communities, which would avoid any uncertainty associated with potential community shifts.

This experiment further strengthens the message portrayed in Chapter 4, by demonstrating that the community phenotype has a significant impact on the rate of pathogen decay. It has also shown that irrespective of differences in other abiotic parameters such as pH or organic carbon, previously regarded as highly influential in pathogen survival, soil biology, specifically the community context, when considered

simultaneously with such factors, is dominant. This has important implications for future predictive modelling and risk assessment of pathogen survival and transmission potential, as it highlights the need to include a measure of community composition.

7.3. Experimental considerations

Community characterisation was carried out based on phenotypic rather than genetic analysis throughout this work. It was decided that the community phenotype would be the most informative measure, as it represented the environmental expression of the genotype, and the immediate construct to which the pathogen was exposed (see above). Thus, pathogen response was assessed primarily on the basis of interactions with the phenotype, rather than the genotype. The phenotype can also be used to discriminate community differences between treatments (Bossio et al., 1998; Frostegård et al., 1997), and this was required to test the hypotheses in Chapters 4, 5 and 6. However, as different microbial species can contain the same fatty acid signature, the phenotype does not provide information at species level. Thus it cannot be used to derive diversity indices, or draw conclusions on species evenness and abundance (Frostegård et al., 2010). Therefore, nucleic acid-based methods such as DGGE and terminal restriction length fragment polymorphism (T-RFLP), which offer more taxonomic resolution, may have added an extra dimension to this study (Zhang and Xu, 2008). For instance, genetic information could have been used to clarify differences in community complexity in Chapter 4, to describe diversity associated with different land-uses in Chapter 5, and to identify species associated with the unintended community shift observed in Chapter 6. It could have also been used to identify microorganisms within phenotypes associated with pathogen suppression. Future work should combine phenotypic with genotypic profiling to provide a comprehensive assessment of the soil microbial community.

Another caveat of the methodological approach adopted throughout this work was the viable but not culturable (VBNC) phenomenon. Cells recovered by culture may not accurately represent the actual concentration of viable cells persisting in the soil over time. This is because microorganisms can enter a dormant state that render them unculturable

when conditions are unfavourable; however they remain viable and can regain culturability when conditions improve (McDougald et al., 1998). Human pathogens have been reported to enter the VBNC when exposed to stress, including *Salmonella enteritidis* (Roszak et al., 1984), *E. coli* (Xu et al., 1982) and *L. monocytogenes* (Colburn et al., 1992). This occurrence may result in underestimation of pathogen survival capability using this method of detection. Again, the use of molecular methods for real-time pathogen quantification such as quantitative PCR (qPCR) may help to overcome this issue. However there are also problems associated with the nucleic acid-based approach. PCR amplification of extracted DNA can result in overestimation of persistence, as DNA is extracted from both live and dead cells, and in free extracellular form. This can be overcome by extracting RNA, which undergoes more rapid turnover in soil and thus represents the living fraction. In any case, both DNA and RNA are typically fraught by technical problems associated with extraction inefficiency and sample inhibition during PCR amplification (Hirsch et al., 2010). Culture work was prescribed as the preferred method of quantification in this instance as it is arguably fit-for-purpose and was straightforward to apply in a high-throughput manner. Also, any potential inaccuracy here caused by VBNC phenomena would have likely been consistent throughout the experiments, and thus would not affect the integrity of data interpretation.

Both culture dependent and independent methods have associated advantages and drawbacks, and ideally, a combination of both would be preferable. However, these experiments were limited by time and financial constraints and thus it was necessary to pick the best tools for the job. After careful consideration, it was decided that culture-based methods for pathogen quantification were the most appropriate to address the hypotheses in question.

7.4. Practical implications and future research

This work has answered questions about the relative importance of the soil microbial community in pathogen suppression. It was shown that viable pathogen residence times in soil are partly contingent on microbial community configuration, suggesting that some

measure of the community should be encompassed in modelling pathogen behaviour with a view to improving the accuracy of risk assessment. The community effect is largely context-dependent, and thus the community should be profiled on a site-specific basis. This information could be used to accurately estimate the time intervals required following livestock manure application before affected sites can be grazed or harvested safely. It could also be used to determine the risk of pathogen loss to nearby watercourses, which could potentially reduce the likelihood of transmission to humans and re-infection of livestock. We now know that pathogen risk may vary between sites, depending on the prevailing phenotype/community context. This is challenging in policy terms since it means that no simple prescription or definition can necessarily be applied to assess risk.

However it now remains to determine the individual species that form suppressive configurations. Therefore, future research should aim to identify these species configurations, and how they interact with introduced pathogens. Additionally a means of manipulating the microbial community to promote these configurations should also be investigated. This may be achieved by inoculation of soil or livestock manures with suppressive cultures that would compete with the pathogen and accelerate pathogen decay. However, this approach is typically associated with variable response and limited success. Alternatively, there is evidence to suggest that organic amendments can potentially enhance pathogen suppression, and it has been hypothesised that this is linked to the quality of the amendment with respect to the C/N ratio (Erickson et al., 2009; Franz et al., 2008b). High quality manure typically contains a high C/N ratio, which is associated with complex carbon sources. Carbon in this form is not readily available to pathogenic microorganisms, which are typically fast-growing opportunists. Rather, slow growing K-strategists utilise this carbon, and thus the pathogen cannot persist. Further work is required to identify the suppressive configurations associated with the manipulation of organic amendments. Notably, in this study, all pathogens were introduced to soils in the absence of any supplementary C, and this was deliberately so to provide the experimentally clearest context to explore the fundamental hypotheses which were set.

In addition, more comparative work on pathogen survival behaviour is required, to ensure that different responses to the community context are taken into account. This

information would enable a more accurate estimation of time to decay for different pathogen types. There is also scope for research on linking survival characteristics to pathogen-specific genetic composition and virulence factors.

It was proven difficult to directly link pathogen survival to communities associated with land-use in this instance. This is because the effects of land-use on community composition are attributed to a complex combination of factors, including management regimes, predominant soil type, and potential legacy effects, all of which are site-specific. This makes it difficult to disentangle the effects of land-use alone. It also makes it difficult to compare results from different studies, and thus draw conclusions. This is technically challenged by the sheer number of circumstances (e.g. pathogen:land-use) which need to be simultaneously characterised in order to provide a robust basis. Further work is necessary to investigate this relationship, perhaps using a stratified approach.

This work has shown that pathogen survival was less related to the absolute number of different species of microbial groups present (i.e. species richness), but rather to what species or groups were present and the shape that they took. Interestingly, this observation is becoming increasingly prevalent in above-ground systems. It is well documented that the diversity of native plant communities is important in providing ecosystem functions and preventing the establishment of invasive plant species (Grime, 1998; Reich et al., 2001; Kennedy et al., 2002). However, studies now show that plant community composition plays an equally important role in maintaining such functions and limiting invisibility potential. Diáz and Cabido (2001) argue that the functional attributes of a plant community are more influential to ecosystem processes than species richness. Work by De Deyn et al., (2009) showed that species richness was positively correlated with C and N dynamics in a model grassland system, however, functionality was associated with the co-occurrence of specific plant species, and thus contingent on species composition rather than absolute diversity. Similarly, plant diversity had no significant effect on soil respiration rates; rather, respiration was driven primarily by the composition of the plant community (Johnson et al., 2008). Additionally, increasing plant diversity resulted in a corresponding decrease in invasibility; however again, this was linked to the presence of specific plant species.

Removal of these species resulted in an increase in susceptibility to invasion. (van Ruijven et al., 2003). Future work in above- and below-ground system should further investigate this link between the community context and ecosystem function.

7.5. Conclusions

This thesis has provided strong evidence to show that soil biology, specifically the *phenotypic community context*, determines pathogen survival behaviour. Therefore a measure of the soil community should be included in modelling pathogen decay for the purposes of risk assessment. Phenotypic profiling provides a possible means to assess the inherent potential of soils to regulate pathogen survival. In addition, it has shown that the precise nature of such survival may be associated with pathogen type. This suggests that the response of different organisms should be taken into account. Future work should focus on identifying specific configurations that are antagonistic toward human pathogens in soil. Research should investigate means of managing the soil in such a way as to allow phenotypic configurations appropriate to attenuate pathogens to be established. This would encourage a greater rate of pathogen decay in soil, which would reduce the risk of pathogen loss to water and crops, and thus break the cycle of infection, leading to better animal and public health protection.

Reference List

Acea M.J., Moore C.R., Alexander M. (1988) Survival and growth of bacteria introduced into soil. *Soil Biology and Biochemistry* 20:509-515.

ADAS. (2001) Safe Sludge Matrix - Guidelines for the application of sewage sludge to agricultural land, UK.

Alef K., Nannipieri, P. (1995) *Methods in Applied Soil Microbiology and Biochemistry*. Academic Press, London.

Allen M.J., Edberg S.C., Reasoner D.J. (2004) Heterotrophic plate count bacteria—what is their significance in drinking water? *International Journal of Food Microbiology* 92:265-274.

Allen M.F., Klironomos J.N., Treseder K.K., Oechel W.C. (2005) Responses of soil biota to elevated CO_2 in a chaparral ecosystem. *Ecological Applications* 15:1701-1711.

Anderson J.P.E., Domsch K.H. (1973) Quantification of bacterial and fungal contributions to soil respiration. *Archives of Microbiology* 93:113-127.

Anderson J.P.E., Domsch K.H. (1978) A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biology and Biochemistry* 10:215-221.

Arrus K.M., Holley R.A., Ominski K.H., Tenuta M., Blank G. (2006) Influence of temperature on *Salmonella* survival in hog manure slurry and seasonal temperature profiles in farm manure storage reservoirs. *Livestock Science* 102:226-236.

ASTM Standard D422-63 (2007) Standard test method for particle-size analysis of soils. ASTM International, West Conshohocken, PA, 2007.

Atwill E.R. (1996) Assessing the link between rangeland cattle and water-borne *Cryptosporidium parvum* infection in humans. *Rangelands* 18:48-51.

Aumaitre A. (1999) Quality and safety of animal products. *Livestock Production Science* 59:113-124.

Austin H.K., Hartel P.G., Coleman D.C. (1990) Effect of genetically-altered *Pseudomonas solanacearum* on predatory protozoa. *Soil Biology and Biochemistry* 22:115-117.

Avery L.M., Hill P., Killham K., Jones D.L. (2004) *Escherichia coli* O157 survival following the surface and sub-surface application of human pathogen contaminated organic waste to soil. *Soil Biology and Biochemistry* 36:2101-2103.

Avery L.M., Killham K., Jones D.L. (2005) Survival of *E. coli* O157:H7 in organic wastes destined for land application. *Journal of Applied Microbiology* 98:814-822.

Bailey V.L., Peacock A.D., Smith J.L., Bolton H. (2002) Relationships between soil microbial biomass determined by chloroform fumigation-extraction, substrate-induced respiration, and phospholipid fatty acid analysis. *Soil Biology and Biochemistry* 34:1385-1389.

Bailey V.L., Smith, J.L., Bolton, H.J. (2007) Substrate-induced respiration and selective inhibition as measures of microbial activity in soils, in: M. R. a. G. Carter, E.G. (Ed.), *Soil Sampling and Methods of Analysis*, Canadian Society of Soil Science, Ottawa, Canada. pp. 515-526.

Bardgett R.D. (2005) *The Biology of Soil - A Community and Ecosystems Approach*. Oxford University Press.

Barker J., Humphrey T.J., Brown M.W.R. (1999) Survival of *Escherichia coli* O157 in a soil protozoan: Implications for disease. *FEMS Microbiology Letters* 173:291-295.

Bell T.H., Klironomos J.N., Henry H.A.L. (2010) Seasonal responses of extracellular enzyme activity and microbial biomass to warming and nitrogen addition. *Soil Science Society of America Journal* 74:820-828.

Benjamin M.M., Datta A.R. (1995) Acid tolerance of enterohemorrhagic *Escherichia coli*. *Applied and Environmental Microbiology* 61:1669-72.

Benskin C.M.H., Wilson K., Jones K., Hartley I.R. (2009) Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biological Reviews* 84:349-373.

Berg G., Eberl L., Hartmann A. (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environmental Microbiology* 7:1673-1685.

Berner A., Hilderman I., Fließbach A., Pfiffner L., Niggli U., Mäder P. (2008) Crop yield and soil fertility response to reduced tillage under organic management. *Soil and Tillage Research* 101:89-96.

Bernet N., Béline F. (2009) Challenges and innovations on biological treatment of livestock effluents. *Bioresource Technology* 100:5431-5436.

Besnard V., Federighi M., Cappelier J.M. (2000) Development of a direct viable count procedure for the investigation of VBNC state in Listeria monocytogenes. *Letters in Applied Microbiology* 31:77-81.

Bethony J., Brooker S., Albonico M., Geiger S.M., Loukas A., Diemert D., Hotez P.J. (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *The Lancet* 367:1521-1532.

Bicudo J.R., Goyal S.M. (2003) Pathogens and manure management systems: A review. *Environmental Technology* 24:115-130.

Blanc C., Sy M., Djigal D., Brauman A., Normand P., Villenave C. (2006) Nutrition on bacteria by bacterial-feeding nematodes and consequences on the structure of soil bacterial community. *European Journal of Soil Biology* 42:S70-S78.

Bligh E.G., Dyer W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37:911-917.

Böhmer M.M., Remschmidt C., Wilking H., Deleré Y., an der Heiden M., Adlhoch C., Dreesman J., Ehlers J., Ethelberg S., Faber M., Frank C., Fricke G., Greiner M., Höhle M., Ivarsson S., Jark U., Kirchner M., Koch J., Krause G., Luber P., Rosner B., Stark K., Kühne M. (2011) German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *New England Journal of Medicine* 365:1763-1770.

Bolton D.J., Byrne C.M., Sheridan J.J., McDowell D.A., Blair I.S. (1999) The survival characteristics of a non-toxigenic strain of *Escherichia coli* O157:H7. *Journal of Applied Microbiology* 86:407-411.

Bolton D.J., Kelly S., Lenahan M., Fanning S. (2011) In-vitro studies on the effect of pH and volatile fatty acid concentration, as influenced by diet, on the survival of inoculated nonacid- and acid-adpated *Salmonella* in bovine rumen fluid and feces. *Foodborne Pathogens and Disease* 8:609-614.

Borneman J., Becker J.O. (2007) Identifying microorganisms involved in specific pathogen suppression in soil. *Annual Review of Phytopathology* 45:153-172.

Bossio D.A., Scow K.M., Gunapala N., Graham K.J. (1998) Determinants of soil microbial communities: Effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. *Microbial Ecology* 36:1-12.

Brake M., Höper H., Joergensen R.G. (1999) Land use-induced changes in activity and biomass of microorganisms in raised bog peats at different depths. *Soil Biology and Biochemistry* 31:1489-1497.

Bray H.R., Kurtz L.T. (1945) Determination of total, organic and available forms of phosphorus in soils. *Soil Science* 59:39-45.

Brennan F.P., Abram F., Chinalia F.A., Richards K.G., O'Flaherty V. (2010a) Characterization of environmentally persistent *Escherichia coli* isolates leached from an Irish soil. *Applied and Environmental Microbiology* 76:2175-2180.

Brennan F.P., O'Flaherty V., Kramers G., Grant J., Richards K.G. (2010b) Long-term persistence and leaching of *Escherichia coli* in temperate maritime soils. *Applied and Environmental Microbiology* 76:1449-1455.

Brewer A.J., Cumby T.R., Dimmock S.J. (1999) Dirty water from dairy farms, II: Treatment and disposal options. *Bioresource Technology* 67:161-169.

Briolat V., Reysset G. (2002) Identification of the *Clostridium perfringens* genes involved in the adaptive response to oxidative stress. *Journal of Bacteriology* 184:2333-2343.

Bronick C.J., Lal R. (2005) Soil structure and management: A review. *Geoderma* 124:3-22.

Brookes P.C., McGrath S.P. (1984) Effect of metal toxicity on the size of the microbial biomass. *Journal of Soil Science* 35:341-346.

Brookes P.C. (2001) The soil microbial biomass: concept, measurement and applications in soil ecosystem research. *Microbes and Environments* 16:131-140.

BSI. (1997) BS 7755-part 4.4.2. Determination of soil microbial biomass – fumigation-extraction methods. Milton Keynes: BSI, 1997.

Buckley D., Schmidt T. (2001) The structure of microbial communities in soil and the lasting impact of cultivation. *Microbial Ecology* 42:11-21.

Bundt M., Widmer F., Pesaro M., Zeyer J., Blaser P. (2001) Preferential flow paths: Biological 'hot spots' in soils. *Soil Biology and Biochemistry* 33:729-738.

Burton C.H. (1992) A review of the strategies in the aerobic treatment of pig slurry: Purpose, theory and method. *Journal of Agricultural Engineering Research* 53:249-272.

Burton C.H., Turner C. (2002) *Manure Management: Treatment Strategies for Sustainable Agriculture*. Second Ed., Silsoe Research Institute.

Byappanahalli M., Fujioka R. (2004) Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. *Water Science and Technology* 50:27-32.

Campbell G.R., Prosser J., Glover A., Killham K. (2001) Detection of *Escherichia coli* O157:H7 in soil and water using multiplex PCR. *Journal of Applied Microbiology* 91:1004-1010.

Chanway C.P., Hynes R.K., Nelson L.M. (1989) Plant growth-promoting rhizobacteria: Effects on growth and nitrogen fixation of lentil (*Lens esculenta* Moench) and pea (*Pisum sativum* L.). *Soil Biology and Biochemistry* 21:511-517.

Chapman P.A., Siddons C.A., Cerdan Malo A.T., Harkin M.A. (1997) A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. *Epidemiology and Infection* 119:245-250.

Chase-Topping M.E., McKendrick I.J., Pearce M.C., MacDonald P., Matthews L., Halliday J., Allison L., Fenlon D., Low J.C., Gunn G., Woolhouse M.E.J. (2007) Risk factors for the presence of high-level shedders of *Escherichia coli* O157 on Scottish farms. *Journal of Clinical Microbiololgy* 45:1594-1603.

Chye F.Y., Abdullah A., Ayob M.K. (2004) Bacteriological quality and safety of raw milk in Malaysia. *Food Microbiology* 21:535-541.

Coleman D.C., Crossley D.A., Hendrix D.F. (2004) *Fundamentals of Soil Ecology*. 2nd Ed. Elsevier Academic Press, San Diego, CA.

Cookson W.R., Murphy D.V., Roper M.M. (2008) Characterizing the relationships between soil organic matter components and microbial function and composition along a tillage disturbance gradient. *Soil Biology and Biochemistry* 40:763-777.

Cooley M.B., Chao D., Mandrell R.E. (2006) *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *Journal of Food Protection* 69:2329-2335.

Cools D., Merckx R., Vlassak K., Verhaegen J. (2001) Survival of *E. coli* and *Enterococcus* spp. derived from pig slurry in soils of different texture. *Applied Soil Ecology* 17:53-62.

Cools I., Uyttendaele M., Caro C., D'Haese E., Nelis H.J., Debevere J. (2003) Survival of *Campylobacter jejuni* strains of different origin in drinking water. *Journal of Applied Microbiology* 94:886-892.

Costa R., Götz M., Mrotzek N., Lottmann J., Berg G., Smalla K. (2006) Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology* 56:236-249.

Côté C., Massé D.I., Quessy S. (2006) Reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion in swine slurries. *Bioresource Technology* 97:686-691.

Crampin M., Willshaw G., Hancock R., Djuretic T., Elstob C., Rouse A., Cheasty T., Stuart J. (1999) Outbreak of *Escherichia coli* O157 infection associated with a music festival. *European Journal of Clinical Microbiology and Infectious Diseases* 18:286-288.

Crawford J.W., Deacon L., Grinev D., Harris J.A., Ritz K., Singh B.K., Young I. (2011). Microbial diversity affects self-organisation of the soil-microbe system with consequences for function. *Journal of the Royal Society Interface* doi:10.1098/rsif.2011.0679.

Cray W.C., Jr., Moon H.W. (1995) Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Applied and Environmental Microbiology* 61:1586-1590.

Curtis T.P., Sloan W.T. (2005) Exploring microbial diversity – A vast below. *Science* 309:1331-1333.

Dahnke W.C (1990) Testing soils for available nitrogen. p. 120-140. *In* Westerman (ed.) Soil testing and plant analysis. Soil Science Society of America Book Series 3, ASA, Madison, WI.

Davies E. (1973) Loss-on-ignition as an estimate of soil organic matter. *Soil Science Society of America Journal* 38:150-151.

Davies C.M., Logan M.R., Rothwell V.J., Krogh M., Ferguson C.M., Charles K., Deere D.A., Ashbolt N.J. (2006) Soil inactivation of DNA viruses in septic seepage. *Journal of Applied Microbiology* 100:365-374.

Davis M.A., Cloud-Hansen K.A., Carpenter J., Hovde C.J. (2005) *Escherichia coli* O157:H7 in environments of culture-positive cattle. *Applied and Environmental Microbiology* 71:6816-6822.

De Deyn, G.B., Quirk, H., Yi, Z., Oakley, S., Ostle, N.J., Bardgett, R. (2009) Vegetation composition promotes carbon and nitrogen storage in model grassland communities of contrasting fertility. *Journal of Ecology* 97:864-875.

De Mesel I., Derycke S., Moens T., Van Der Gucht K., Vincx M., Swings J. (2004) Top-down impact of bacterivorous nematodes on the bacterial community structure: A microcosm study. *Environmental Microbiology* 6:733-744.

DEFRA. (2010) Fertiliser recommendations for Agricultural and Horticultural Crops (RB209), 8th Ed., The Stationary Office, London

Degens B.P., Schipper L.A., Sparling G.P., Vojvodic-Vukovic M. (2000) Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. *Soil Biology and Biochemistry* 32:189-196.

Deng M.Y., Cliver D.O. (1992) Inactivation of poliovirus Type 1 in mixed human and swine wastes and by bacteria from swine manure. *Applied and Environmental Microbiology* 58:2016-2021.

Diáz S., Cabido M. (2001) Vive la différence: plant functional diversity matters to ecosystem services. *Trends in Ecology and Evolution* 16:646-655.

Diez-Gonzalez F., Callaway T.R., Kizoulis M.G., Russell J.B. (1998) Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science* 281:1666-1668.

Djigal D., Brauman A., Diop T.A., Chotte J.L., Villenave C. (2004) Influence of bacterial-feeding nematodes (*Cephalobidae*) on soil microbial communities during maize growth. *Soil Biology and Biochemistry* 36:323-331.

Dowd S.E., Callaway T.R., Morrow-Tesch J. (2007) Handling may cause increased shedding of *Escherichia coli* and total coliforms in pigs. *Foodborne Pathogens and Disease* 4:99-102.

Drake, E.H., Motto, H.L. (1982) An Analysis of the Effect of Clay and Organic Matter Content on the Cation Exchange Capacity of New Jersey Soils. *Soil Science* 133:281-288.

Duffy G. (2003) Verocytoxigenic *Escherichia coli* in animal faeces, manures and slurries. *Journal of Applied Microbiology* 94:94-103.

EC. (1986) Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture.

EC. (1991) Council Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources.

EC. (2001) S.I. No. 267/2001 — Waste Management (Use of Sewage Sludge in Agriculture) (Amendment) Regulations, 2001.

EC. (2010) S.I. No. 610/2010 — European Communities (Good Agricultural Practice for Protection of Waters) Regulations 2010.

EFSA. (2007) Scientific opinion of the panel on biological hazards on a request from EFSA on monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC types. *The EFSA Journal* 2007:1-61.

EFSA. (2011) Shiga toxin-producing *E. coli (*STEC) O104:H4 2011 outbreaks in Europe: Taking Stock. *The EFSA Journal* 9:2390-2412.

England L.S., Lee H., Trevors J.T. (1993) Bacterial survival in soil: Effect of clays and protozoa. *Soil Biology and Biochemistry* 25:525-531.

Erickson M.C., Liao J., Ma L., Jiang X., Doyle M.P. (2009) Inactivation of *Salmonella* spp. in cow manure composts formulated to different initial C:N ratios. *Bioresource Technology* 100:5898-5903.

Esperschütz J., Gattinger A., Mäder P., Schloter M., Fließbach A. (2007) Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. *FEMS Microbiology Ecology* 61:26-37.

FAO. (2006) World reference base for soil resources, Report No. 103, Rome.

Feng Y., Motta A.C., Reeves D.W., Burmester C.H., van Santen E., Osborne J.A. (2003) Soil microbial communities under conventional-till and no-till continuous cotton systems. *Soil Biology and Biochemistry* 35:1693-1703.

Fenlon D.R., Ogden I.D., Vinten A., Svoboda I. (2000) The fate of *Escherichia coli* and *E. coli* O157 in cattle slurry after application to land. Symposium series (*Society for Applied Microbiology*):149S-156S.

Fierer N., Jackson R.B. (2006) The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences* 3:626-631.

Forslund A., Markussen B., Toenner-Klank L., Bech T.B., Jacobsen O.S., Dalsgaard A. (2011) Leaching of *Cryptosporidium parvum* oocysts, *Escherichia coli*, and a *Salmonella enterica* Serovar Typhimurium Bacteriophage through intact soil cores following surface application and injection of slurry. *Applied and Environmental Microbiology* 77:8129-8138.

Frank C., Faber M.S., Askar M., Bernard H., Fruth A., Gilsdorf A., Höhle M., Karch M., Krause G., Prager R., Spode A., Stark K., Werber D. (2011) Large and ongoing outbreak of haemolytic uraemic syndrome, Germany, May 2011. *Euro Surveillance* 16:2-4.

Franz E., Semenov A., van Bruggen A. (2008a) Modelling the contamination of lettuce with *Escherichia coli* O157:H7 from manure-amended soil and the effect of intervention strategies. *Journal of Applied Microbiology* 105:1569-1584.

Franz E., Semenov A.V., Termorshuizen A.J., De Vos O.J., Bokhorst J.G., van Bruggen A.H.C. (2008b) Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils. *Environmental Microbiology* 10:313-327.

Franz E., van Diepeningen A.D., De Vos O.J., van Bruggen A.H.C. (2005) Effects of cattle feeding regimen and soil management type on the fate of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in manure, manure-amended soil, and lettuce. *Applied and Environmental Microbiology* 71:6165-6174.

Franz E., van Hoek A.H.A.M., Bouw E., Aarts H.J.M. (2011) *E. coli* strain variability in manure-amended soil in relation to strain origin, virulence profile and carbon nutrition profile. *Applied and Environmental Microbiology* 77:8088-8096.

Frostegård Å., Bååth E., Tunlio A. (1993) Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. *Soil Biology and Biochemistry* 25:723-730.

Frostegård Å., Petersen S.O., Bååth E., Nielsen T.H. (1997) Dynamics of a microbial community associated with manure hot spots as revealed by phospholipid fatty acid analyses. *Applied and Environmental Microbiology* 63:2224-2231.

Frostegård Å., Tunlid A., Bååth E. (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Applied and Environmental Microbiology*. 59:3605-3617.

Frostegård Å., Tunlid A., Bååth E. (1991) Microbial biomass measured as total lipid phosphate in soils of different organic content. *Journal of Microbiological Methods* 14:151-163.

Frostegård Å., Tunlid A., Bååth E. (2010) Use and misuse of PLFA measurements in soils. *Soil Biology and Biochemistry* In Press, Corrected Proof.

FSAI. (1999) The prevention of *E. Coli* O157:H7 infection: A shared responsibility, Dublin, Ireland.

Gadre R.V., Ranade D.R., Godbole S.H. (1986) A note on survival of salmonellas during anaerobic digestion of cattle dung. *Journal of Applied Microbiology* 60:93-96.

Gagliardi J.V., Karns J.S. (2002) Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. *Environmental Microbiology* 4:89-96.

Gans J., Wolinsky M., Dunbar J. (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* 309:1387-1390.

Garbeva P., van Veen J.A., van Elsas J.D. (2004a) Assessment of the diversity, and antagonism towards *Rhizoctonia solani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. *FEMS Microbiology Ecology* 47:51-64.

Garbeva P., van Veen J.A., van Elsas J.D. (2004b) Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annual Review of Phytopathology* 42:243-270.

Garcia M.M., McKay K.A. (1970) Pathogenic microorganisms in soil: An old problem in a new perspective. *Canadian Journal of Comparative Medicine* 34:105-110.

Gerba C.P., Melnick J.L., Wallis C. (1975) Fate of wastewater bacteria and viruses in soil. *Journal of the Irrigation and Drainage Division* 101:157-174.

Gessel P.D., Hansen N.C., Goyal S.M., Johnston L.J., Webb J. (2004) Persistence of zoonotic pathogens in surface soil treated with different rates of liquid pig manure. *Applied Soil Ecology* 25:237-243.

Gilbert R.A., Tomkins N., Padmanabha J., Gough J.M., Krause D.O., McSweeney C.S. (2005) Effect of finishing diets on *Escherichia coli* populations and prevalence of enterohaemorrhagic *E. coli* virulence genes in cattle faeces. *Journal of Applied Microbiology* 99:885-894.

Girvan M.S., Bullimore J., Pretty J.N., Osborn A.M., Ball A.S. (2003) Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. *Applied and Environmental Microbiology* 69:1800-1809.

Girvan M.S., Campbell C.D., Killham K., Prosser J.I., Glover L.A. (2005) Bacterial diversity promotes community stability and functional resilience after perturbation. *Environmental Microbiology* 7:301-313.

Gorissen A., van Elsas J.D. (2004) Pig slurry reduces the survival of *Ralstonia solanacearum* biovar 2 in soil. *Canadian Journal of Microbiology* 50:587-593.

Gotz A., Smalla K. (1997) Manure enhances plasmid mobilization and survival of *Pseudomonas putida* Introduced into field soil. *Applied and Environmental Microbiology* 63:1980-6.

Gourabathini P., Brandi M.T., Redding K.S., Gunderson J.H., Berk S.G. (2008) Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Applied and Environmental Microbiology* 74:2518-2525.

Govaerts B., Mezzalama M., Unno Y., Sayre K.D., Luna-Guido M., Vanherck K., Dendooven L., Deckers J. (2007) Influence of tillage, residue management, and crop rotation on soil microbial biomass and catabolic diversity. *Applied Soil Ecology* 37:18-30.

Graham M.H., Haynes R.J. (2005) Catabolic diversity of soil microbial communities under sugarcane and other land uses estimated by Biolog and substrate-induced respiration methods. *Applied Soil Ecology* 29:155-164.

Grewal S., Sreevatsan S., Michel Jr F.C. (2007) Persistence of *Listeria* and *Salmonella* during swine manure treatment. *Compost Science and Utilization* 15:53-62.

Griffiths B.S. (1994) Microbial-feeding nematodes and protozoa in soil: Their effects on microbial activity and nitrogen mineralization in decomposition hotspots and the rhizosphere. *Plant and Soil* 164:25-33.

Griffiths B.S., Ritz K., Bardgett R.D., Cook R., Christensen S., Ekelund F., Sørensen S.J., Bååth E., Bloem J., De Ruiter P.C., Dolfing J., Nicolardot B. (2000) Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity–ecosystem function relationship. *Oikos* 90:279-294.

Griffiths B.S., Ritz K., Wheatley R., Kuan H.L., Boag B., Christensen S., Ekelund F., Sørensen S.J., Muller S., Bloem J. (2001) An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. *Soil Biology and Biochemistry* 33:1713-1722.

Griffths B.S., Kuan H.L., Ritz K., Glover L.A., McCaig A.E., Fenwick C. (2004) The relationship between microbial community structure and functional stability, tested experimentally in an upland pasture soil. *Microbial Ecology* 47:104-113.

Griffiths B., Hallett P., Kuan H., Gregory A., Watts C., Whitmore A. (2008) Functional resilience of soil microbial communities depends on both soil structure and microbial community composition. *Biology and Fertility of Soils* 44:745-754.

Grime J.P. (1998) Benefits of plant diversity to ecosystems: immediate, filter and founder effects. *Journal of Ecology*, 86:902-910.

Guardabassi L., Dalsgaard A., Sobsey M. (2003) Occurence and survival of viruses in composted human faeces, *Sustainable Urban Renewal and Wastewater Treatment*, Danish EPA.

Habteselassie M., Bischoff M., Blume E., Applegate B., Reuhs B., Brouder S., Turco R.F. (2008) Environmental controls on the fate of *Escherichia* coli in soil. *Water, Air, and Soil Pollution* 190:143-155.

Haichar F.e.Z., Marol C., Berge O., Rangel-Castro J.I., Prosser J.I., Balesdent J., Heulin T., Achouak W. (2008) Plant host habitat and root exudates shape soil bacterial community structure. *ISME* 2:1221-1230.

Hao X., Chang C., Larney F.J., Travis G.R. (2001) Greenhouse gas emissions during cattle feedlot manure composting. *Journal of Environmental Quality* 30:376-386.

Hartmann M., Fliessbach A., Oberholzer H.R., Widmer F. (2006) Ranking the magnitude of crop and farming system effects on soil microbial biomass and genetic structure of bacterial communities. *FEMS Microbiology Ecology* 57:378-388.

Hartmann M., Widmer F. (2006) Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. *Applied and Environmental Microbiology* 72:7804-7812.

Heijnen C.E., van Veen J.A. (1991) A determination of protective microhabitats for bacteria introduced into soil. *FEMS Microbiology Letters* 85:73-80.

Heinonen-Tanski H., Mohaibes M., Karinen P., Koivunen J. (2006) Methods to reduce pathogen microorganisms in manure. *Livestock Science* 102:248-255.

Hernández D., Hobbie S. (2010) The effects of substrate composition, quantity, and diversity on microbial activity. *Plant and Soil* 335:397-411.

Heuvelink A.E., van Heerwaarden C., Zwartkruis-Nahuis J., T, M, van Oosterom R., Edink K., van Duynhoven Y., H, P, De Boer E. (2002a) *Escherichia coli* O157 infection associated with a petting zoo. *Epidemiology and Infection* 129:295-302.

Himathongkham S., Bahari S., Riemann H., Cliver D. (1999) Survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in cow manure and cow manure slurry. *FEMS Microbiology Letters* 178:251-257.

Himathongkham S., Riemann H. (1999) Destruction of *Salmonella* Typhimurium, *Escherichia coli* O157:H7 and *Listeria monocytogenes* in chicken manure by drying and/or gassing with ammonia. *FEMS Microbiology Letters* 171:179-182.

Hirsch P.R., Mauchline T.H., Clark I.M. (2010) Culture-independent molecular techniques for soil microbial ecology. *Soil Biology and Biochemistry* 42:878-887.

Ho W.C., Ko W.H. (1985) Soil microbiostasis: Effects of environmental and edaphic factors. *Soil Biology and Biochemistry* 17:167-170.

Holley R.A., Arrus K.M., Ominski K.H., Tenuta M., Blank G. (2006) *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. *Journal of Environmental Quality* 35:1170-1180.

Hovde C.J., Austin P.R., Cloud K.A., Williams C.J., Hunt C.W. (1999) Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. *Applied and Environmental Microbiology* 65:3233-3235.

Hoxie N.J., Davis J.P., Vergeront J.M., Nashold R.D., Blair K.A. (1997) Cryptosporidiosis-associated mortality following a massive waterborne outbreak in Milwaukee, Wisconsin. *American Journal of Public Health* 87:2032-2035.

Hrudey S.E., Payment P., Huck P.M., Gillham R.W., Hrudey E.J. (2003) A fatal waterborne disease epidemic in Walkerton, Ontario: Comparison with other waterborne outbreaks in the developed world. *Water Science and Technology* 47:7-14.

Hurst C.J., Gerba C.P., Cech I. (1980) Effects of environmental variables and soil characteristics on virus survival in soil. *Applied and Environmental Microbiology* 40:1067-1079.

Hutchison M.L., Walters L.D., Avery S.M., Synge B.A., Moore A. (2004a) Levels of zoonotic agents in British livestock manures. *Letters in Applied Microbiology* 39:207-214.

Hutchison M.L., Walters L.D., Moore A., Crookes K.M., Avery S.M. (2004b) Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. *Applied and Environmental Microbiology* 70:5111-5118.

Hyde B., Carton O.T. (2005) Manure management facilities on farms and their relevance to efficient nutrient use., *Fertiliser Association of Ireland*, Winter Scientific Meeting, UCD, Dublin, Ireland.

Hyde B.P., Carton O.T., O'Toole P., Misselbrook T.H. (2003) A new inventory of ammonia emissions from Irish agriculture. *Atmospheric Environment* 37:55-62.

Ibekwe A.M., Papiernik S.K., Grieve C.M., Yang C.H. (2010) Influence of fumigants on soil microbial diversity and survival of *E. coli* O157:H7. *Journal of Environmental Science and Health*, Part B: Pesticides, Food Contaminants, and Agricultural Wastes 45:416 - 426.

Islam M., Doyle M.P., Phatak S.C., Millner P., Jiang X. (2005) Survival of *Escherichia coli* O157:H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. *Food Microbiology* 22:63-70.

ISO. (1997) 14240-1: *Soil quality part 1: Determination of soil microbial biomass – Substrate induced respiration method*. International Standards Organisation.

Itoh Y., Sugita-Konishi Y., Kasuga F., Iwaki M., Hara-Kudo Y., Saito N., Noguchi Y., Konuma H., Kumagai S. (1998) Enterohemorrhagic *Escherichia coli* O157:H7 present in radish sprouts. *Applied and Environmental Microbiology* 64:1532-1535.

Jamieson R.C., Gordon R.J., Sharples K.E., Stratton G.W., Madani A. (2002) Movement and persistence of fecal bacteria in agricultural soils and sub-surface drainage water: A review. *Canadian Biosystems Engineering* 44:1-1.9.

Jangid K., Williams M.A., Franzluebbers A.J., Sanderlin J.S., Reeves J.H., Jenkins M.B., Endale D.M., Coleman D.C., Whitman W.B. (2008) Relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems. *Soil Biology and Biochemistry* 40:2843-2853.

Jeffery S., Harris J.A., Rickson R.J., Ritz K. (2007) Microbial community phenotypic profiles change markedly with depth within the first centimetre of the arable soil surface. *Soil Biology and Biochemistry* 39:1226-1229.

Jenkins M.B., Bowman D.D., Fogarty E.A., Ghiorse W.C. (2002) *Cryptosporidium parvum* oocyst inactivation in three soil types at various temperatures and water potentials. *Soil Biology and Biochemistry* 34:1101-1109.

Jenkins M.B., Walker M.J., Bowman D.D., Anthony L.C., Ghiorse W.C. (1999) Use of a sentinel system for field measurements of *Cryptosporidium parvum* oocyst inactivation in soil and animal waste. *Applied and Environmental Microbiology* 65:1998-2005.

Jenkinson D.S., Powlson D.S. (1976a) The effects of biocidal treatments on metabolism in soil--I. Fumigation with chloroform. *Soil Biology and Biochemistry* 8:167-177.

Jiang S., Chu W., Olson B., He J.-W., Choi S., Zhang J., Le J., Gedalanga P. (2007) Microbial source tracking in a small southern California urban watershed indicates wild animals and growth as the source of fecal bacteria. *Applied Microbiology and Biotechnology* 76:927-934.

Jiang X., Morgan J., Doyle M.P. (2002) Fate of *Escherichia coli* O157:H7 in manure-amended soil. *Applied and Environmental Microbiology* 68:2605-2609.

Joergensen R.G., Wu J., Brookes P.C. (2011) Measuring soil microbial biomass using an automated procedure. *Soil Biology and Biochemistry* 43:873-876.

Johnson, D., Phoenix, G.K., Grime, J.P. (2008) Plant community composition, not diversity, regulates soil respiration in grasslands. *Biology Letters* 4:345-348

Jones D.L. (1999) Potential health risks associated with the persistence of *Escherichia coli* O157 in agricultural environments. *Soil Use and Management* 15:76-83.

Jones K. (2001) Campylobacters in water, sewage and the environment. *Journal of Applied Microbiology* 90:68S-79S.

Jones K., Howard S., Wallace J.S. (1999) Intermittent shedding of thermophilic campylobacters by sheep at pasture. *Journal of Applied Microbiology* 86:531-536.

Jousset A., Scheu S., Bonkowski M. (2008) Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. *Functional Ecology* 22:714-719.

Kahn K.S., Heinze S., Joergensen R.G. (2009) Simultaneous measurement of S, macronutrients, and heavy metals in the soil microbial biomass with $CHCl_3$ fumigation and NH_4NO_3 extraction. *Soil Biology and Biochemistry* 41:309-314.

Kaur A., Chaudhary, A., Kaur, A., Choudhary, A., Kaushik, R. (2005) Phospholipid fatty acid – A bioindicator of environment monitoring and assessment in soil ecosystem. *Current Science* 89:1103-1102.

Keel C., Ucurum Z.h., Michaux P., Adrian M., Haas D. (2002) Deleterious impact of a virulent bacteriophage on survival and biocontrol activity of *Pseudomonas fluorescens* strain CHA0 in natural soil. *Molecular Plant-Microbe Interactions* 15:567-576.

Keeley A., Faulkner B.R. (2008) Influence of land use and watershed characteristics on protozoa contamination in a potential drinking water resources reservoir. *Water Research* 42:2803-2813.

Kennedy T.A., Naeem S., Howe K.M., Knops J.M.H., Tilman D., Reich P. (2002) Biodiversity as a barrier to ecological invasion. *Nature* 417:636-638.

Kibblewhite M.G., Ritz K., Swift M.J. (2008) Soil health in agricultural systems. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363:685-701.

Kielak A., Pijl A.S., Van Veen J.A., Kowalchuk G.A. (2008) Differences in vegetation composition and plant species identity lead to only minor changes in soil-borne microbial communities in a former arable field. *FEMS Microbiology Ecology* 63:372-382.

Kimura M., Jia Z.J., Nakayama N., S. A. (2008) Ecology of viruses in soils: Past, present and future perspectives. *Soil Science and Plant Nutrition* 54:1-32.

Koch A.L. (1994) Growth measurement. In: Gerhardt, P. Ed., Methods for General and Molecular Batcteriology. ASM Press, Washington, DC, 255.

Kok C.J., Hageman P.E.J., Maas P.W.T., Postma J., Roozen N.J.M., Van Vuurde J.W.L. (1996) Processed manure as carrier to introduce *Trichoderma harzianum*: Population dynamics and biocontrol effect on *Rhizoctonia solani*. *Biocontrol Science and Technology* 6:147-161.

Kudva I.T., Hunt C.W., Williams C.J., Nance U.M., Hovde C.J. (1997) Evaluation of dietary influences on *Escherichia coli* O157:H7 shedding by sheep. *Applied and Environmental Microbiology* 63:3878-3886.

Kudva I.T., Jelacic S., Tarr P.I., Youderian P., Hovde C.J. (1999) Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. *Applied and Environmental Microbiology* 65:3767-3773.

Kutílek, M. (2004) Soil hydraulic properties as related to soil structure. *Soil Tillage and Research* 79:175-184.

Lang N.L., Smith S.R. (2007) Influence of soil type, moisture content and biosolids application on the fate of *Escherichia coli* in agricultural soil under controlled laboratory conditions. *Journal of Applied Microbiology* 103:2122-2131.

Lartey R.T. (2006) Dynamics of soil flora and fauna in biological control of soil inhabiting plant pathogens. *Plant Pathology Journal* 5:125-142.

Lauber C.L., Hamady M., Knight R., Fierer N. (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology* 75:5111-5120.

Lauber C.L., Strickland M.S., Bradford M.A., Fierer N. (2008) The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biology and Biochemistry* 40:2407-2415.

Leifert C., Ball K., Volakakis N., Cooper J.M. (2008) Control of enteric pathogens in ready-to-eat vegetable crops in organic and 'low input' production systems: A HACCP-based approach. *Journal of Applied Microbiology* 105:931-950.

Lejon D., Sebastia J., Lamy I., Chaussod R., Ranjard L. (2007) Relationships between soil organic status and microbial community density and genetic structure in two agricultural soils submitted to various types of organic management. *Microbial Ecology* 53:650-663.

Liang Z., He Z., Powell C.A., Stoffella P.J. (2011) Survival of *Escherichia coli* in soil with modified microbial community composition. *Soil Biology and Biochemistry* 43:1591-1599.

Lin J., Smith M.P., Chapin K.C., Baik H.S., Bennett G.N., Foster J.W. (1996) Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Applied and Environmental Microbiology* 62:3094-3100.

Mac Kenzie W.R., Hoxie N.J., Proctor M.E., Gradus M.S., Blair K.A., Peterson D.E., Kazmierczak J.J., Addiss D.G., Fox K.R., Rose J.B., Davis J.P. (1994) A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *New England Journal of Medicine* 331:161-167.

Malik Y.S., Randall, G.W., Goyal, S.M. (2004) Fate of *Salmonella* following application of swine manure to tile-drained clay loam soil. *Journal of Water and Health* 2: 97-101.

March S.B., Ratnam S. (1986) Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *Journal of Clinical Microbiology* 23:869-872.

Matos A., Kerkhof L., Garland J.L. (2005) Effects of microbial community diversity on the survival of *Pseudomonas aeruginosa* in the wheat rhizosphere. *Microbial Ecology* 49:257-264.

Maul J., Drinkwater L. (2010) Short-term plant species impact on microbial community structure in soils with long-term agricultural history. *Plant and Soil* 330:369-382.

Mawdsley J.L., Bardgett R.D., Merry R.J., Pain B.F., Theodorou M.K. (1995) Pathogens in livestock waste, their potential for movement through soil and environmental pollution. *Applied Soil Ecology* 2:1-15.

Mazzola M. (2002) Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 81:557-564.

McDougald D., Rice S.A., Weichart D., Kjelleberg S. (1998) Nonculturability: adaptation or debilitation? *FEMS Microbiology Ecology* 25:1-9.

McGrady-Steed J., Harris P.M., Morin P.J. (1997) Biodiversity regulates ecosystem predictability. *Nature* 390:162-165.

McGeehan S.L., Naylor D.V. (1988) Automated instrumental analysis of carbon and nitrogen in plant and soil samples. *Communications in Soil Science and Plant Analysis* 19:493-505.

McLean E.O. (1982). Soil pH and lime requirement. p. 199-223. *In* A.L. Page et al., (eds.) *Methods of Soil Analysis, Part 2*. Agronomy Monographs 9, 2nd ed. ASA and SSSA, Madison, WI.

McNamara N.P., Black H.I.J., Beresford N.A., Parekh N.R. (2003) Effects of acute gamma irradiation on chemical, physical and biological properties of soils. *Applied Soil Ecology* 24:117-132.

Mehlich A. (1984) Mehlich-3 soil test extractant: A modification of Mehlich-2 extractant. *Communications in Soil Science and Plant Analysis* 15:1409-1416.

Mikkelsen L.L., Naughton P.J., Hedemann M.S., Jensen B.B. (2004) Effects of physical properties of feed on microbial ecology and survival of *Salmonella enterica* serovar Typhimurium in the pig gastrointestinal tract. *Applied and Environmental Microbiology* 70:3485-3492.

Mohaibes M., Heinonen-Tanski H. (2004) Aerobic thermophilic treatment of farm slurry and food wastes. *Bioresource Technology* 95:245-254.

Morales V.L., Parlange J.Y., Steenhuis T.S. (2010) Are preferential flow paths perpetuated by microbial activity in the soil matrix? A review. *Journal of Hydrology* 393:29-36.

Morris J.G., Potter M. (1997) Emergence of new pathogens as a function of changes in host susceptibility. *Emerging Infectious Diseases* 3:433-41.

Mubiru D.N., Coyne M.S., Grove J.H. (2000) Mortality of *Escherichia coli* O157:H7 in two soils with different physical and chemical properties. *Journal of Environmental Quality* 29:1821-1825.

Mukherjee A., Speh D., Dyck E., Diez-Gonzalez F. (2004) Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in organic and conventional produce grown by Minnesota farmers. *Journal of Food Protection* 67:894-900.

Naeem S., Li S. (1997) Biodiversity enhances ecosystem reliability. *Nature* 390:507-509.

Nasser A.M., Glozman R., Nitzan Y. (2002) Contribution of microbial activity to virus reduction in saturated soil. *Water Research* 36:2589-2595.

Nelson D.W., Sommers L.E. (1996) Total carbon, organic carbon and organic matter. p. 961-1010. *In* J.M. Bartels et al., (ed.) *Methods of Soil Analysis: Part 3 Chemical methods*. (3rd ed.) ASA and SSSA Book Series 5, Madison, WI.

Nicholson F.A., Chambers B.J., Moore A., Nicholson R.J., Hickman G. (2004) Assessing and managing the risks of pathogen transfer from livestock manures into the food chain. *Water and Environment Journal* 18:155-160.

Nicholson F.A., Groves S.J., Chambers B.J. (2005) Pathogen survival during livestock manure storage and following land application. *Bioresource Technology* 96:135-143.

Nielsen E.M., Tegtmeier C., Andersen H.J., Grønbæk C., Andersen J.S. (2002) Influence of age, sex and herd characteristics on the occurrence of verocytotoxin-producing *Escherichia coli* O157 in Danish dairy farms. *Veterinary Microbiology* 88:245-257.

Nyberg K.A., Vinnerås B., Ottoson J.R., Aronsson P., Albihn A. (2010) Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in manure-amended soils studied in outdoor lysimeters. *Applied Soil Ecology* 46:398-404.

O'Flynn G., Ross R.P., Fitzgerald G.F., Coffey A. (2004) Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology* 70:3417-3424.

Ogden I.D., Fenlon D.R., Vinten A.J.A., Lewis D. (2001) The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. *International Journal of Food Microbiology* 66:111-117.

Ogden I.D., Hepburn N.F., MacRae M., Strachan N.J.C., Fenlon D.R., Rusbridge S.M., Pennington T.H. (2002) Long-term survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. *Letters in Applied Microbiology* 34:100-104.

Ogden I.D., MacRae M., Strachan N.J. (2004) Is the prevalence and shedding concentrations of *E. coli* O157 in beef cattle in Scotland seasonal? *FEMS Microbiology Letters* 233:297-300.

Oliver D.M., Haygarth P.M., Clegg C.D., Heathwaite A.L. (2006) Differential *E. coli* die-off patterns associated with agricultural matrices. *Environmental Science and Technology* 40:5710-5716.

Olsen S.R., Sommers L.E. (1982) Phosphorus. p. 403-430. In A.L. Page et al., (eds.) *Methods of Soil Analysis, Part 2*. Agronomy Monographs 9 2nd ed. ASA and SSSA, Madison, WI.

Olsen J.E., Larsen H.E. (1987) Bacterial decimation times in anaerobic digestions in animal slurries. *Biological Wastes* 21:153-168.

Olson M.E., Goh J., Phillips M., Guselle N., McAllister T.A. (1999) *Giardia* cyst and *Cryptosporidium parvum* oocyst survival in water, soil and cattle feces. *Applied and Environmental Microbiology* 55:1223-1229.

Olszewska H., Zbigniew P., Jarzabek Z. (2008) Survival of bovine enterovirus strain LCR-4 in water, slurry and soil. *Bull Vet Inst Pulawy* 52:205-209.

Østergård H., Finckh M.R., Fontaine L., Goldringer I., Hoad S.P., Kristensen K., Lammerts van Bueren E.T., Mascher F., Munk L., Wolfe M.S. (2009) Time for a shift in crop production: embracing complexity through diversity at all levels. *Journal of the Science of Food and Agriculture* 89: 1439-1445.

Parashar U.D., Gibson C.J., Bresse J.S., Glass R.I. (2006) Rotavirus and severe childhood diarrhea. *Emerging infectious diseases* 12:304-6.

Patra A.K., Le Roux X., Grayston S.J., Loiseau P., Louault F. (2008) Unraveling the effects of management regime and plant species on soil organic carbon and microbial phospholipid fatty acid profiles in grassland soils. Bioresource Technology 99:3545-3551.

Pell A.N. (1997) Manure and microbes: public and animal health problem? *Journal of Dairy Science* 80:2673-2681.

Pelly H., Cormican M., O'Donovan D., Chalmers R.M., Hanahoe B., Cloughley R., McKeown P., Corbett-Feeney G. (2007) A large outbreak of cryptosporidiosis in western Ireland linked to public water supply: A preliminary report. *Euro Surveillance* 12:3187.

Pennie R.A., Zunino J.N., Rose C.E., Guerrant R.L. (1984) Economical, simple method for production of the gaseous environment required for cultivation of *Campylobacter jejuni*. *Journal of Clinical Microbiology* 20:320-322.

Petersen S.O., Klug M.J. (1994) Effects of sieving, storage, and incubation temperature on the phospholipid fatty acid profile of a soil microbial community. *Applied and Environmental Microbiology* 60:2421-2430.

Porter J., Mobbs K., Hart C.A., Saunders J.R., Pickup R.W., Edwards C. (1997) Detection, distribution and probable fate of *Escherichia coli* 0157 from asymptomatic cattle on a dairy farm. *Journal of Applied Microbiology* 83:297-306.

Postma J., Hok-Hin A.C.H., van Veen J.A. (1990) Role of microniches in protecting introduced *Rhizobium leguminosarum* biovar Trifolii against competition and predation in soil. *Applied and Environmental Microbiology* 56:495-502.

Postma J., van Veen J.A., Walter S. (1989) Influence of different initial soil moisture contents on the distribution and population dynamics of introduced *Rhizobium leguminosarum* biovar Trifolii. *Soil Biology and Biochemistry* 21:437-442.

Raaijmakers J.M., Vlami M., de Souza J.T. (2002) Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 81:537-547.

Raaijmakers J.M., Weller D.M. (1998) Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Molecular Plant-Microbe Interactions* 11:144-152.

Ramette A., Moënne-Loccoz Y., Défago G. (2003) Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS Microbiology Ecology* 44:35-43.

Ramirez N.E., Ward L.A., Sreevatsan S. (2004) A review of the biology and epidemiology of cryptosporidiosis in humans and animals. *Microbes and Infection* 6:773-785.

Ramos M.C., Quinton J.N., Tyrrel S.F. (2006) Effects of cattle manure on erosion rates and runoff water pollution by faecal coliforms. *Journal of Environmental Management* 78:97-101.

Rattray E.A., Prosser J.I., Killham K., Glover L.A. (1990) Luminescence-based nonextractive technique for in situ detection of *Escherichia coli* in soil. *Applied and Environmental Microbiology* 56:3368-3374.

Razavi S., Lakzian A. (2007) Evaluation of chemical and biological consequences of soil sterilisation methods. *Caspian Journal of Environmental Science* 5:87-91.

Recorbet G., Steinberg C., Faurie G. (1992) Survival in soil of genetically engineered *Escherichia coli* as related to inoculum density, predation and competition. *FEMS Microbiology Ecology* 101:251-260.

Reddy K.R., Khaleel R., Overcash M.R. (1981) Behavior and transport of microbial pathogens and indicator organisms in soils treated with organic wastes. *Journal of Environmental Quality* 10:255-266.

Reich, P.B., Knops, J., Tilman, D., Craine, J., Ellsworth, D., Tjoelker, M., Lee, T., Wedin, D., Naeem, S., Bahauddin, D., Hendrey, G., Jose, S., Wragel, K., Goth, J. and Bengston, W. (2001) Plant diversity enhances ecosystem responses to elevated CO_2 and nitrogen deposition. *Nature*, 410:809-810.

Ritz K. (2005) Underview: origins and consequences of below ground biodiversity. *In* Biological Diversity and Function of Soils, Cambridge Univeristy Press. pp. 381-401.

Ritz K. (2007) The plate debate: Cultivable communities have no utility in contemporary environmental microbial ecology. *FEMS Microbiology Ecology* 60:358-362.

Rivera-Cruz M.d.C., Trujillo Narcía A., Córdova Ballona G., Kohler J., Caravaca F., Roldán A. (2008) Poultry manure and banana waste are effective biofertilizer carriers for promoting plant growth and soil sustainability in banana crops. *Soil Biology and Biochemistry* 40:3092-3095.

Robertson L.J., Gjerde B.K. (2004) Effects of the Norwegian winter environment on *Giardia* cysts and *Cryptosporidium* oocysts. *Microbial Ecology* 47:359-365.

Rodhe L., Pell M., Yamulki S. (2006) Nitrous oxide, methane and ammonia emissions following slurry spreading on grassland. *Soil Use and Management* 22:229-237.

Rodríguez H., Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* 17:319-339.

Rondon M.R., Goodman R.M., Handelsman J. (1999) The Earth's bounty: assessing and accessing soil microbial diversity. *Trends in Biotechnology* 17:403-409.

Rose J.B., Gerba C.P., Jakubowski W. (1991) Survey of potable water supplies for *Cryptosporidium* and *Giardia*. *Environmental Science and Technology* 25:1393-1400.

Rosen B. (2000) Waterborne pathogens in agricultural watersheds, USDA, NRCS, Watershed Science Intsitute 1-62.

Ross D. (1995) Recommended soil tests for determining exchange capacity. p. 62-69. *In* Sims J.T., Wolf A. (eds.) Recommended soil testing procedures for the northeastern

United States. Northeastern Regional Bulletin No. 493. Agricultural Experiment Station, University of Delaware, Newark, DE.

Ross D.J., Speir T.W., Kettles H.A., Mackay A.D. (1995) Soil microbial biomass, C and N mineralization and enzyme activities in a hill pasture: Influence of season and slow-release P and S fertilizer. *Soil Biology and Biochemistry* 27:1431-1443.

Roszak D.B., Grimes D.J., Colwell R.R. (1984) Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. *Canadian Journal of Microbiology* 30:334-338.

Rugbjerg H., Nielsen E.M., Andersen J.S. (2003) Risk factors associated with faecal shedding of verocytotoxin-producing *Escherichia coli* O157 in eight known-infected Danish dairy herds. *Preventive Veterinary Medicine* 58:101-113.

Russell J.B., Diez-Gonzalez F., Jarvis G.N. (2000) Potential effect of cattle diets on the transmission of pathogenic *Escherichia coli* to humans. *Microbes and Infection* 2:45-53.

Russell J.B., Rychlik J.L. (2001) Factors that alter rumen microbial ecology. *Science* 292:1119-1122.

Rutherford P.M., Juma N.G. (1992) Influence of texture on habitable pore space and bacterial-protozoan populations in soil. *Biology and Fertility of Soils* 12:221-227.

Sagoo S.K., Little C.L., Mitchell R.T. (2001) The microbiological examination of ready-to-eat organic vegetables from retail establishments in the United Kingdom. *Letters in Applied Microbiology* 33:434-439.

Salomonová S., Lamačová, J., Rulík, M., Rolčík, J., Čáp, L., Bednář, P. and Barták, P. (2003) Determination of phospholipid fatty acids in sediments. *AUPO Chemica* 42:39-49.

Santamaría J., Toranzos G.A. (2003) Enteric pathogens and soil: A short review. *International Microbiology* 6:5-9.

Schönfeld J., Gelsomino A., van Overbeek L.S., Gorissen A., Smalla K., van Elsas J.D. (2003) Effects of compost addition and simulated solarisation on the fate of *Ralstonia solanacearum* biovar 2 and indigenous bacteria in soil. *FEMS Microbiology Ecology* 43:63-74.

Schulte E.E., Hopkins B.G. (1996) Estimation of soil organic matter by weight loss-on-ignition. p. 21-32. *In* F.R. Magdoff, M.A. Tabatabai, E.A. Hanlon. *Soil Organic Matter: Analysis and Interpretation*. Special Publication No. 46. Soil Science Society of America, Madison, WI.

Semenov A.V., van Bruggen, A.H.C., van Overbeek, L., Termorshuizen, A.J. and Semenov, A.M. (2007) Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cow manure. *FEMS Microbiology Ecology* 60:419-428.

Semenov A.V., van Overbeek L., Termorshuizen A.J., van Bruggen A.H.C. (2010) Influence of aerobic and anaerobic conditions on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in Luria-Bertani broth, farm-yard manure and slurry. *Journal of Environmental Management* In Press, Corrected Proof.

Semenov A.V., van Overbeek L., van Bruggen A.H.C. (2009) Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Applied and Environmental Microbiology* 75:3206-3215.

Sheng H., Knecht H.J., Kudva I.T., Hovde C.J. (2006) Application of bacteriophages to control intestinal *Escherichia coli* O157:H7 levels in ruminants. *Applied and Environmental Microbiology* 72:5359-5366.

Shiomi Y., Nishiyama M., Onizuka T., Marumoto T. (1999) Comparison of bacterial community structures in the rhizoplane of tomato plants grown in soils suppressive and conducive towards bacterial wilt. *Applied and Environmental Microbiology* 65:3996-4001.

Shukla R., Slack R., George A., Cheasty T., Rowe B., Scutter J. (1995) *Escherichia coli* O157 infection associated with a farm visitor centre. Communicable disease report. *CDR Review* 5:R86-90.

Sidhu J., Gibbs R.A., Ho G.E., Unkovich I. (2001) The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. *Water Research* 35:913-920.

Sjogren R.E. (1994) Prolonged survival of an environmental *Escherchia coli* in laboratory soil microcosms. *Water, Air, and Soil Pollution* 75:389-403.

Sjogren R.E. (1995) Thirteen-year survival study of an environmental *Escherichia coli* in field mini-plots. *Water, Air, and Soil Pollution* 81:315-335.

Smalla K., Wieland G., Buchner A., Zock A., Parzy J., Kaiser S., Roskot N., Heuer H., Berg G. (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* 67:4742-4751.

Smith A., Reacher M., Smerdon W., Adak G.K., Nichols G., Chalmers R.M. (2006) Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992–2003. *Epidemiology and Infection* 134:1141-1149.

Smith K.A., Jackson D.R., Pepper T.J. (2001a) Nutrient losses by surface run-off following the application of organic manures to arable land. 1. Nitrogen. *Environmental Pollution* 112:41-51.

Smith K.A., Jackson D.R., Withers P.J.A. (2001b) Nutrient losses by surface run-off following the application of organic manures to arable land. 2. Phosphorus. *Environmental Pollution* 112:53-60.

Sobsey M.D., Dean C.H., Knuckles M.E., Wagner R.A. (1980) Interactions and survival of enteric viruses in soil materials. *Applied and Environmental Microbiology* 40:92-101.

Söderström A., Österberg P., Lindqvist A., Jönsson B., Lindberg A., Blide Ulander S., Welinder-Olsson C., Löfdahl S., Kaijser B., De Jong B., Kühlmann-Berenzon S., Boqvist S., Eriksson E., Szanto E., Andersson S., Allestam G., Hedenström I., Ledet Muller L., Andersson Y. (2008) A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathogens and Disease* 5:339-349.

Solomon E.B., Yaron S., Matthews K.R. (2002) Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Applied and Environmental Microbiology* 68:397-400.

Sørensen S.J., Schyberg T., Rønn R. (1999) Predation by protozoa on *Escherichia coli* K12 in soil and transfer of resistance plasmid RP4 to indigenous bacteria in soil. *Applied Soil Ecology* 11:79-90.

Sparling G. (1992) Ratio of microbial biomass carbon to soil organic carbon as a sensitive indicator of changes in soil organic matter. *Soil Research* 30:195-207.

Steenwerth K.L., Jackson L.E., Calderón F.J., Stromberg M.R., Scow K.M. (2002) Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. *Soil Biology and Biochemistry* 34:1599-1611.

Steer J., Harris J.A. (2000) Shifts in the microbial community in rhizosphere and non-rhizosphere soils during the growth of *Agrostis stolonifera*. *Soil Biology and Biochemistry* 32:869-878.

Stephens J.H.G., Rask H.M. (2000) Inoculant production and formulation. *Field Crops Research* 65:249-258.

Su C., Brandt L.J. (1995) *Escherichia coli* O157: H7 infection in humans. *Annals of Internal Medicine* 123:698-707.

Takahashi J.A., Castro M.C.M.d., Souza G.G., Lucas E.M.F., Bracarense A.A.P., Abreu L.M., Marriel I.E., Oliveira M.S., Floreano M.B., Oliveira T.S. (2008) Isolation and screening of fungal species isolated from Brazilian cerrado soil for antibacterial activity

against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Streptococcus pyogenes* and *Listeria monocytogenes*. *Journal of Medical Mycology* 18:198-204.

Texier S., Prigent-Combaret C., Gourdon M.H., Poirier M.A., Faivre P., Dorioz J.M., Poulenard J., Jocteur-Monrozier L., Moënne-Loccoz Y., Trevisan D. (2008) Persistence of culturable *Escherichia* coli fecal contaminants in dairy alpine grassland soils. *Journal of Environmental Quality* 37:2299-2310.

Thompson R.C.A., Palmer C.S., O'Handley R. (2008) The public health and clinical significance of *Giardia* and *Cryptosporidium* in domestic animals. *Veterinary Journal* 177:18-25.

Thurston-Enriquez J.A., Gilley J.E., Eghball B. (2005) Microbial quality of runoff following land application of cattle manure and swine slurry. *Journal of Water and Health* 3:157-171.

Tiedje J.M., Asuming-Brempong S., Nüsslein K., Marsh T.L., Flynn S.J. (1999) Opening the black box of soil microbial diversity. *Applied Soil Ecology* 13:109-122.

Tilman D., Balzer C., Hill J., Befort B.L. (2011) Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences* 108:20260-20264.

Topp E., Welsh M., Tien Y.C., Dang A., Lazarovits G., Conn K., Zhu H. (2003) Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiology Ecology* 44:303-308.

Torsvik V., Øvreås L. (2002) Microbial diversity and function in soil: From genes to ecosystems. *Current Opinion in Microbiology* 5:240-245.

Toyota K., Kimura M. (2000) Suppression of *Ralstonia solanacearum* in soil following colonization by other strains of *R. solanacearum*. *Soil Science and Plant Nutrition* 46:449-459.

Toyota K., Kimura M., Kinoshita T. (2000) Microbiological factors affecting the colonization of tomato roots by *Ralstonia solanacearum* YU1Rif43lux. *Soil Science and Plant Nutrition* 46:643-653.

Toyota K., Ritz K., Young I.M. (1996) Microbiological factors affecting the colonisation of soil aggregates by *Fusarium oxysporum f. sp. raphani*. *Soil Biology and Biochemistry* 28:1513-1521.

Tyrrel S.F., Quinton J.N. (2003) Overland flow transport of pathogens from agricultural land receiving faecal wastes. *Journal of Applied Microbiology* 94:87-93.

Unc A., Goss M.J. (2006) Culturable *Escherichia coli* in soil mixed with two types of manure. *Soil Science Society of America Journal* 70:763-769.

Upchurch R., Chiu C.-Y., Everett K., Dyszynski G., Coleman D.C., Whitman W.B. (2008) Differences in the composition and diversity of bacterial communities from agricultural and forest soils. *Soil Biology and Biochemistry* 40:1294-1305.

van Bruggen A.H.C., Semenov A.M. (2000) In search of biological indicators for soil health and disease suppression. *Applied Soil Ecology* 15:13-24.

van Elsas J.D., Garbeva P., Salles J. (2002) Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. *Biodegradation* 13:29-40.

van Elsas J.D., Hill P., Chronakova A., Grekova M., Topalova Y., Elhottova D., Kristufek V. (2007) Survival of genetically marked *Escherichia coli* O157:H7 in soil as affected by soil microbial community shifts. *ISME* 1:204-214.

van Elsas J.D., Semenov A.V., Costa R., Trevors J.T. (2011) Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME* 5:173-183.

van Ruijven, J., De Deyn, G.B., Berendse F. (2003). Diversity reduces invasibility in experimental plant communities: the role of plant species. *Ecology Letters* 6:910-918.

van Veen J.A., van Overbeek L.S., van Elsas J.D. (1997) Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews* 61:121-135.

Vance E.D., Brookes P.C., Jenkinson D.S. (1987) An extraction method for measuring soil microbial biomass C. *Soil Biology and Biochemistry* 19:703-707.

Varel V.H. (2002) Carvacrol and thymol reduce swine waste odor and pathogens: Stability of oils. *Current Microbiology* 44:38-43.

Vargas R., Hattori T. (1986) Protozoan predation of bacterial cells in soil aggregates. *FEMS Microbiology Letters* 38:233-242.

Venette R.C., Ferris H. (1998) Influence of bacterial type and density on population growth of bacterial-feeding nematodes. *Soil Biology and Biochemistry* 30:949-960.

Vidovic S., Block H.C., Korber D.R. (2007) Effect of soil composition, temperature, indigenous microflora, and environmental conditions on the survival of *Escherichia coli* O157:H7. *Canadian Journal of Microbiology* 53:822-829.

Vinnerås B. (2007) Comparison of composting, storage and urea treatment for sanitising of faecal matter and manure. *Bioresource Technology* 98:3317-3321.

Wakelin S.A., Macdonald L.M., Rogers S.L., Gregg A.L., Bolger T.P., Baldock J.A. (2008) Habitat selective factors influencing the structural composition and functional capacity of microbial communities in agricultural soils. *Soil Biology and Biochemistry* 40:803-813.

Waksman S.A., Woodruff H.B. (1940) Survival of bacteria added to soil and resultant modification of the soil population. *Soil Science* 50:421-427.

Wang G., Zhao T., Doyle M.P. (1996) Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Applied and Environmental Microbiology* 62:2567-2570.

Wang W.J., Dalal R.C., Moody P.W., Smith C.J. (2003) Relationships of soil respiration to microbial biomass, substrate availability and clay content. *Soil Biology and Biochemistry* 35:273-284.

Wertz S., Degrange V., Prosser J.I., Poly F., Commeaux C., Freitag T., Guillaumaud N., Roux X.L. (2006) Maintenance of soil functioning following erosion of microbial diversity. *Environmental Microbiology* 8:2162-2169.

West A.W., Sparling G.P. (1986) Modifications to the substrate-induced respiration method to permit measurement of microbial biomass in soils of differing water contents. *Journal of Microbiological Methods* 5:177-189.

White D.C., Davis W.M., Nickels J.S., King J.D., Bobbie R.J. (1979) Determination of the sedimentary microbial biomass by extractible lipid phosphate. *Oecologia* 40:51-62.

WHO. (2006) Microbial Fact Sheets. Guidelines for drinking-water quality, Geneva, Switzerland 222-295.

Williams A.P., Avery L.M., Killham K., Jones D.L. (2005) Persistence of *Escherichia coli* O157 on farm surfaces under different environmental conditions. *Journal of Applied Microbiology* 98:1075-1083.

Williams A.P., Avery L.M., Killham K., Jones D.L. (2007a) Persistence, dissipation, and activity of *Escherichia coli* O157:H7 within sand and seawater environments. *FEMS Microbiology Ecology* 60:24-32.

Williams A.P., Avery L.M., Killham K., Jones D.L. (2007b) Survival of *Escherichia coli* O157:H7 in the rhizosphere of maize grown in waste-amended soil. *Journal of Applied Microbiology* 102:319-326.

Williams A.P., McGregor K.A., Kilham K., Jones D.L. (2008) Persistence and metabolic activity of *Escherichia coli* O157:H7 in farm animal faeces. *FEMS Microbiology Letters* 287:168-173.

Willshaw G., Thirlwell J., Jones A., Parry S., Salmon R., Hickey M. (1994) Vero cytotoxin-producing *Escherichia coli* O157 in beefburgers linked to an outbreak of

diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome in Britain. *Letters in Applied Microbiology* 19:304-307.

Winfield M.D., Groisman E.A. (2003) Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology* 69:3687-3694.

Wright D.A., Killham K., Glover L.A., Prosser J.I. (1995) Role of pore size location in determining bacterial activity during predation by protozoa in soil. *Applied and Environmental Microbiology* 61:3537-3543.

Wu J., Joergensen, R.G., Pommerening, B., Chaussod, R., Brookes P.C. (1990) Measurement of soil microbial biomass C by fumigation-extraction - an automated procedure. *Soil Biology and Biochemistry* 22:1167-1169.

Wu Y., Ding N., Wang G., Xu J., Wu J., Brookes P.C. (2009) Effects of different soil weights, storage times and extraction methods on soil phospholipid fatty acid analyses. *Geoderma* 150:171-178.

Xiao L., Moore J.E., Ukoh U., Gatei W., Lowery C.J., Murphy T.M., Dooley J.S.G., Millar B.C., Rooney P.J., Rao J.R. (2006) Prevalence and identity of *Cryptosporidium* spp. in pig slurry. *Applied and Environmental Microbiology* 72:4461-4463.

Xu H.S., Robert N., Singleton F.L., Attwel R.W., Grimes D.J., Colwell, R.R. (1982) Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecology*, 8:313-323.

Yamada T., Kawasaki T., Nagata S., Fujiwara A., Usami S., Fujie M. (2007) New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. *Microbiology* 153:2630-2639.

Yin C., Jones K.L., Peterson D.E., Garrett K.A., Hulbert S.H., Paulitz T.C. (2010) Members of soil bacterial communities sensitive to tillage and crop rotation. *Soil Biology and Biochemistry* 42:2111-2118.

Yossa N., Patel J., Miller P., Lo Y.M. (2010a) Antimicrobial activity of essential oils against *Escherichia coli* O157:H7 in organic soil. *Food Control* 21:1458-1465.

Yossa N., Patel J., Millner P., Lo M. (2010b) Inactivation of *Salmonella* in organic soil by cinnamaldehyde, eugenol, ecotrol, and sporan. *Foodborne Pathogens and Disease* 8:311-317.

You Y., Rankin S.C., Aceto H.W., Benson C.E., Toth J.D., Dou Z. (2006) Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Applied and Environmental Microbiology* 72:5777-5783.

Zelles L. (1999) Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A review. *Biology and Fertility of Soils* 29:111-129.

Zelles L., Bai Q.Y., Rackwitz R., Chadwick D., Beese F. (1995) Determination of phospholipid- and lipopolysaccharide-derived fatty acids as an estimate of microbial biomass and community structures in soils. *Biology and Fertility of Soils* 19:115-123.

Zhang L., Xu Z. (2008) Assessing bacterial diversity in soil. *Journal of Soils and Sediments* 8:379-388.

Zhang R.H., Westerman P.W. (1997) Solid-liquid separation of animal manure for odor control and nutrient management. *Applied Engineering in Agriculture* 13:657-664.

Zhao B., Zhang H., Zhang J., Jin Y. (2008) Virus adsorption and inactivation in soil as influenced by autochthonous microorganisms and water content. *Soil Biology and Biochemistry* 40:649-659.

Zheng Y., Ge S., Zhang J., Guo Q., Ng M.H., Wang F., Xia N., Jiang Q. (2006) Swine as a principal reservoir of Hepatitis E virus that infects humans in eastern China. *Journal of Infectious Diseases* 193:1643-1649.

Zhou J., Xia B., Treves D.S., Wu L.Y., Marsh T.L., O'Neill R.V., Palumbo A.V., Tiedje J.M. (2002) Spatial and resource factors influencing high microbial diversity in soil. *Applied and Environmental Microbiology* 68:326-334.

Appendix 1

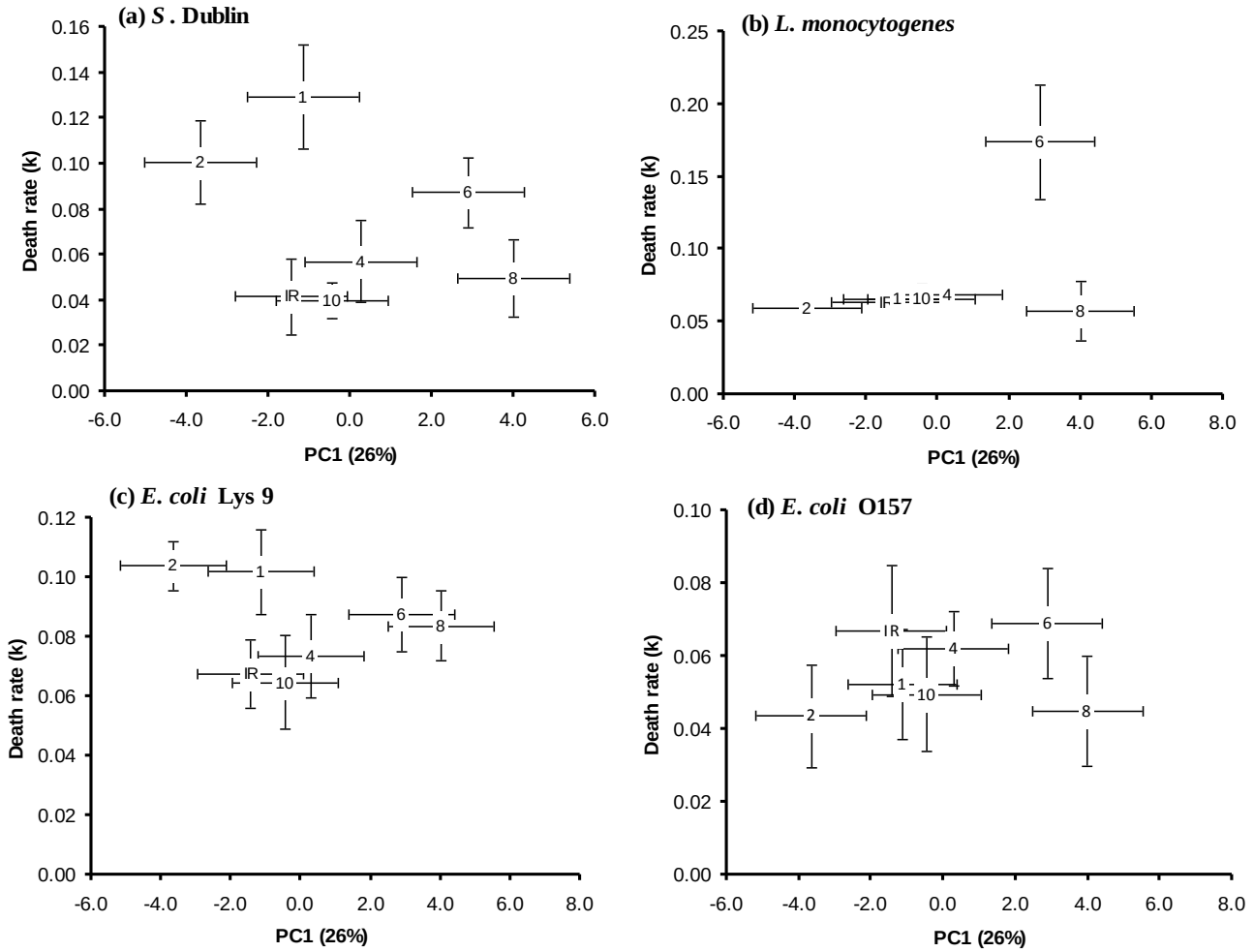


Fig. 1A Relationship between pathogen death rate and first principal component for (a) *S*. Dublin (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4), which fall within confines of symbols for some treatments

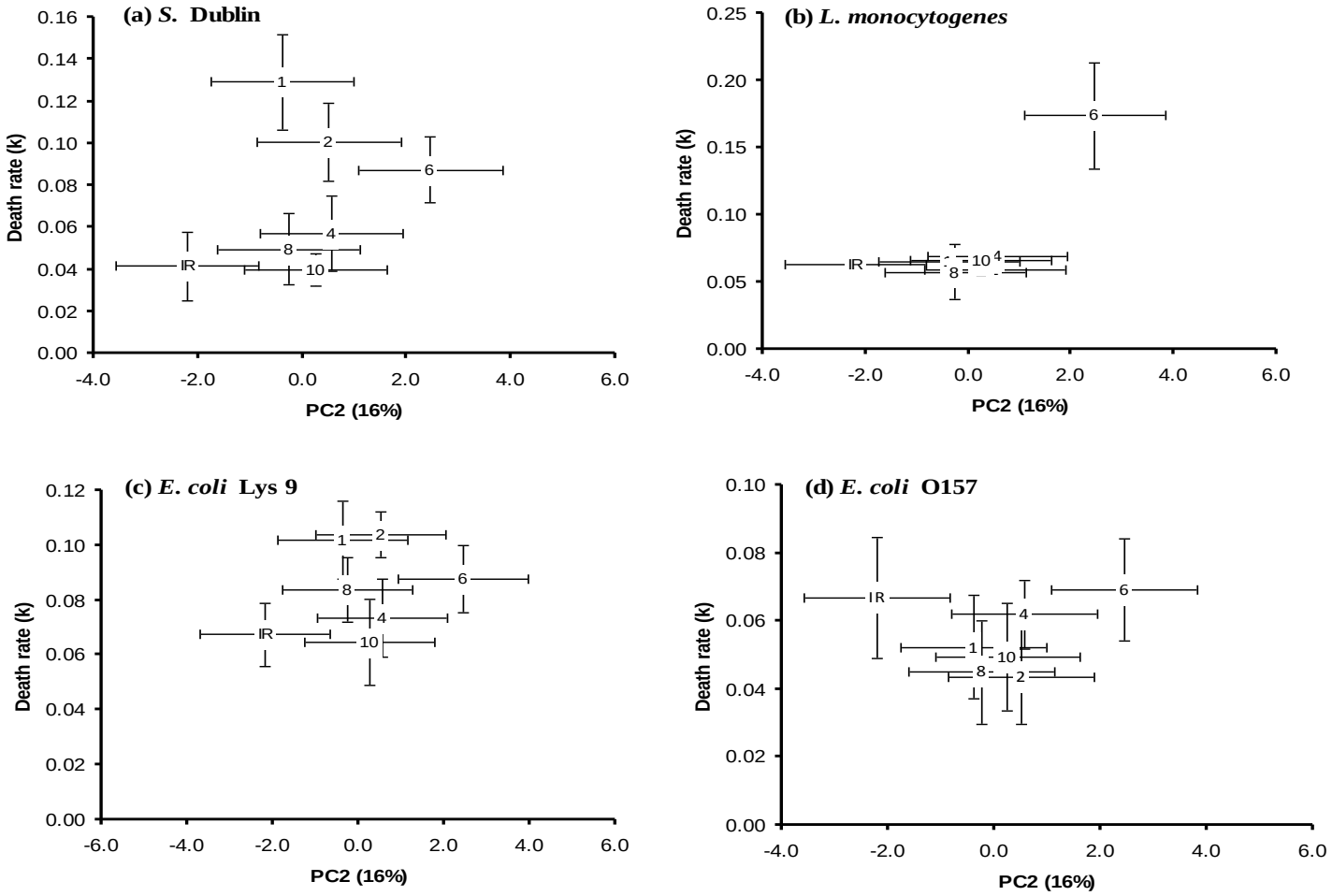


Fig. 1B Relationship between pathogen death rate and second principal component for (a) *S.* Dublin (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4), which fall within confines of symbols for some treatments

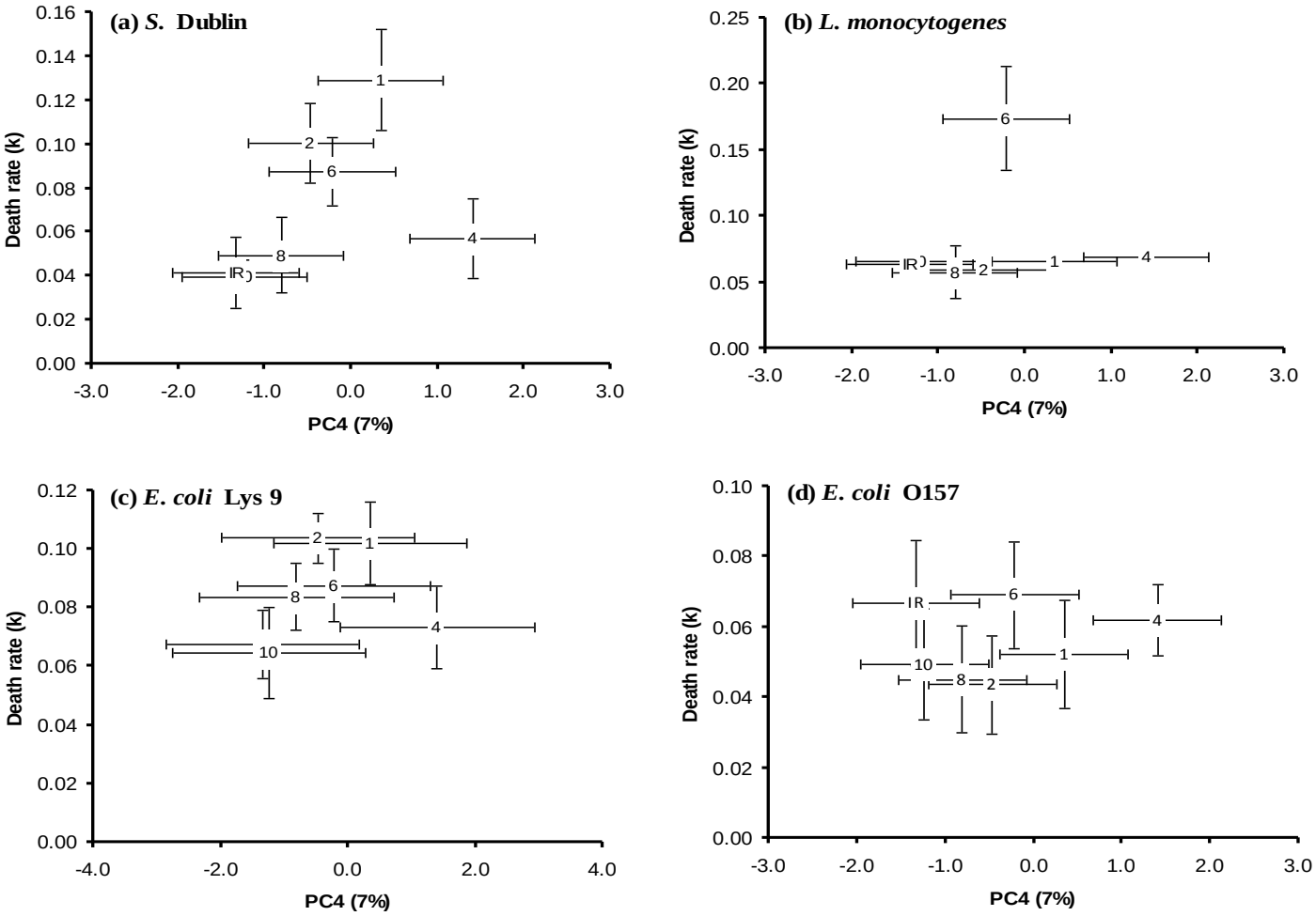


Fig. 1C Relationship between pathogen death rate and fourth principal component for (a) *S*. Dublin (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. 0-10=dilutions 10^0-10^{10}, IR=irradiated control. Error bars represent standard error (n=4), which fall within confines of symbols for some treatments

Appendix 2

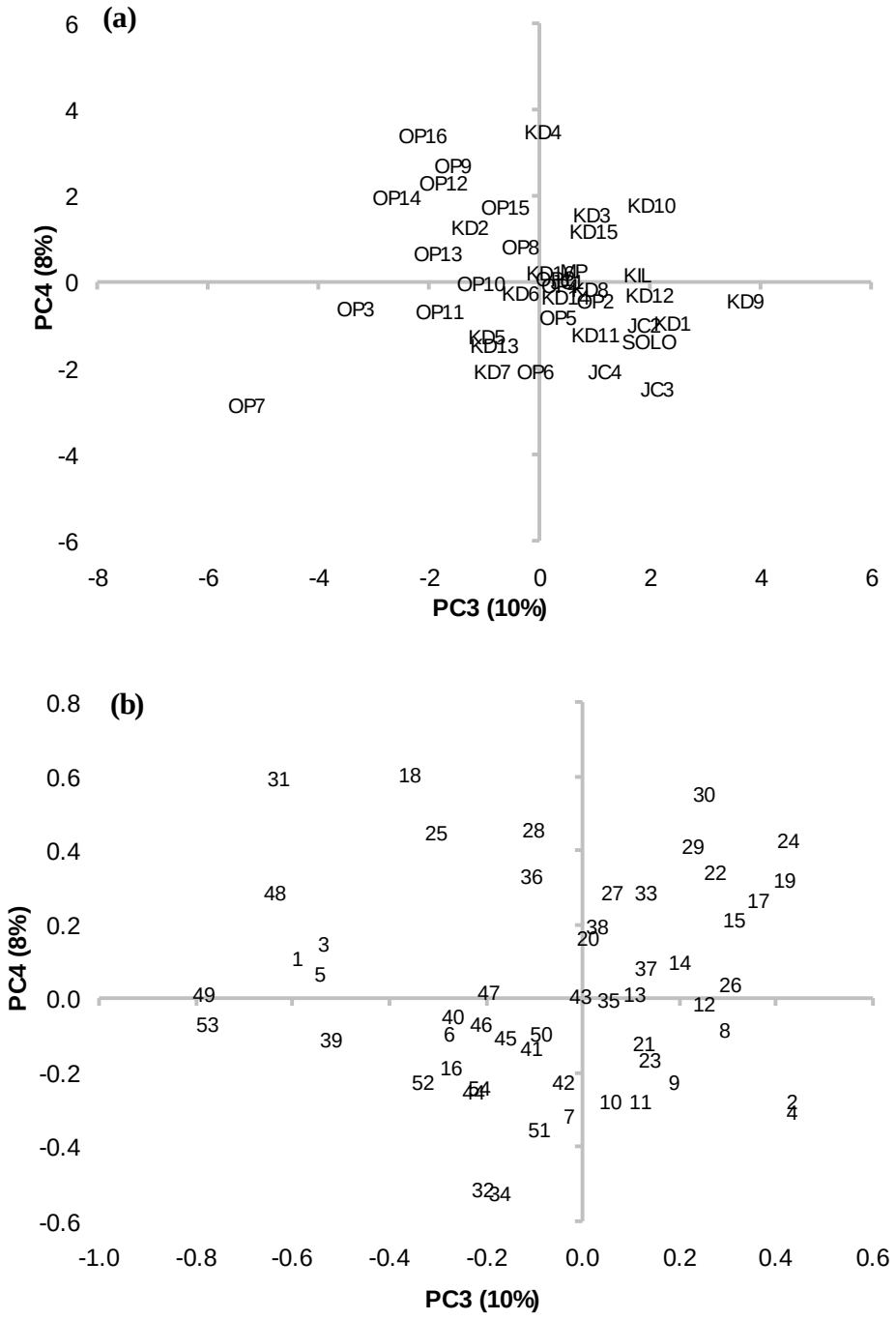


Fig. 2A Third and fourth principal components derived from average PLFA profiles of 39 soils according to soil ID; (b) loading plot showing PLFAs contributing to PC discrimination (n=3) See Table 5.2 for PLFA identification

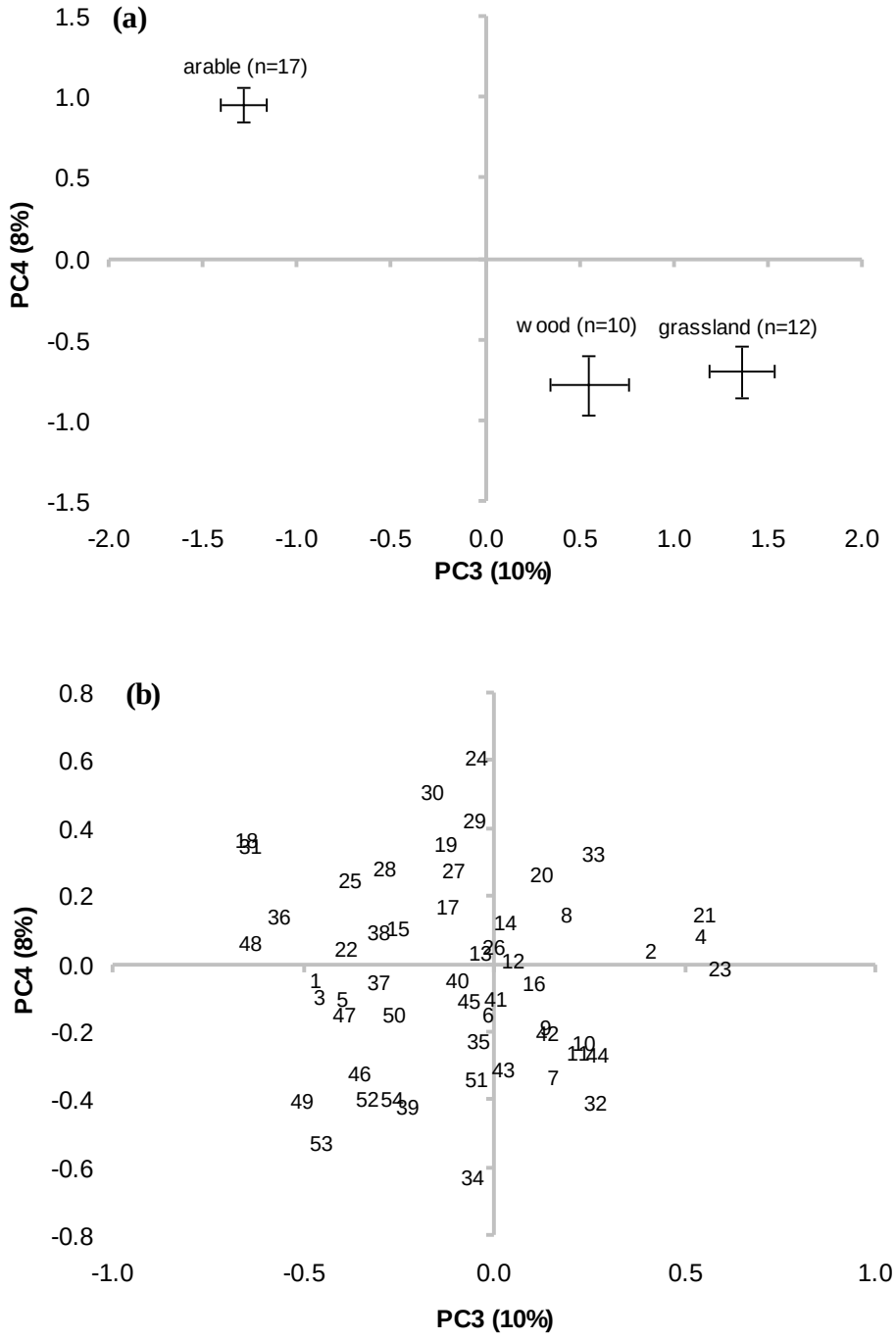


Fig. 2B Third and fourth principal components derived from average PLFA profiles of 39 soils according to (a) average land-use (error bars represent standard error, n shown in parenthesis) and (b) loading plot showing PLFAs contributing to discrimination. See Fig. 5.2 for PLFA identification

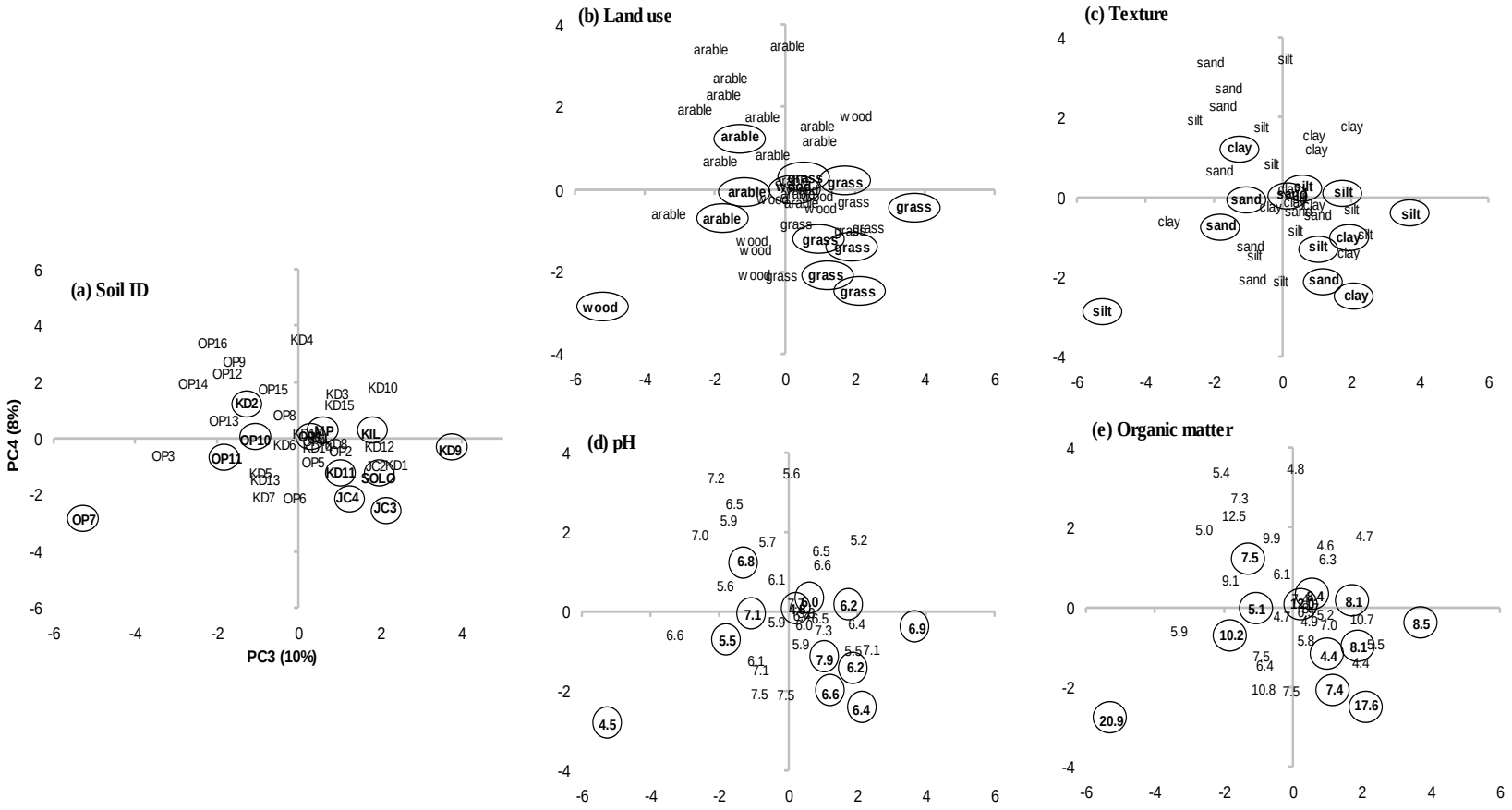


Fig. 2C Third and fourth principal components derived from PLFA profiles of 39 soils according to (a) soil ID, (b) land-use, (c) texture, (d) pH and (e) % OM (n=3). Circled data-points represent final 12 soils selected for pathogen survival analysis

Appendix 3

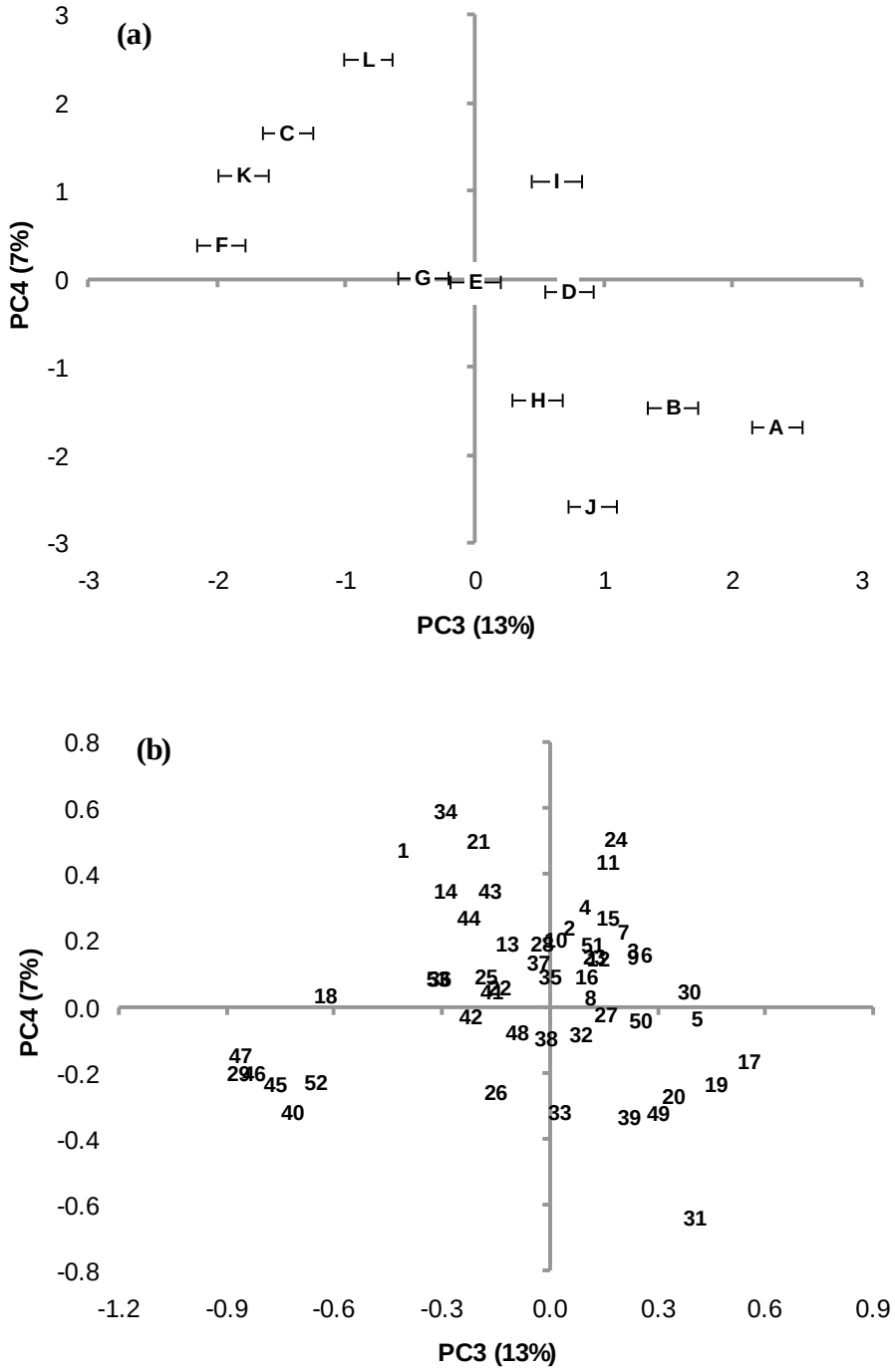


Fig. 3A (a) Ordination of third and fourth principal components derived from average PLFA profiles with respect to soils; (b) Corresponding loading plot showing PLFAs responsible for PC discrimination. Data represent PC scores ± standard error (n=12). See Table 6.1 for soil identification and Table 6.2 for PLFA identification

Table 3A First four PC scores derived from average PLFA profiles for each pathogen batch at respective T_0's

	Batch 1				Batch 2				Batch 3				Batch 4			
	L. monocytogenes				*S.* Dublin				*E. coli* Lys 9				*E. coli* O157			
Soil	PC1* (33%)	PC2* (18%)	PC3* (16%)	PC4* (6%)	PC1* (26%)	PC2* (21%)	PC3^{ns} (14%)	PC4* (9%)	PC1* (28%)	PC2** (22%)	PC3** (11%)	PC4^{ns} (10%)	PC1* (41%)	PC2* (14%)	PC3^{ns} (11%)	PC4** (7%)
JC4	0.40	-1.45	1.97	-1.90	2.61	-0.19	0.50	-2.75	3.56	1.89	1.16	0.74	0.89	0.02	1.64	1.93
JC3	2.74	1.47	2.64	-2.00	1.69	0.31	-0.78	-3.28	0.66	0.30	2.50	3.12	1.68	3.11	0.77	1.65
OP7	2.48	4.62	-1.23	-2.60	-1.03	5.25	0.22	-2.43	3.55	-4.95	1.90	1.55	-5.76	2.15	-1.95	2.41
OP1	4.70	1.87	-1.54	-0.35	1.34	3.60	-0.38	1.96	0.13	-2.92	-0.73	0.64	-1.81	2.05	-1.50	-1.90
KD2	-5.23	0.34	-0.83	0.83	-4.33	-0.88	1.16	1.25	-4.75	0.12	-1.30	-0.25	2.07	-0.57	-1.38	-3.09
KD15	4.14	-1.69	-3.88	1.41	-0.17	3.39	0.12	1.31	-1.23	-3.23	-3.20	-0.33	-3.33	-2.96	-3.82	1.32
KIL	1.89	-4.97	2.03	1.13	2.73	-6.16	1.16	1.21	-0.85	5.40	-0.74	-0.58	6.93	-3.16	0.50	0.54
KD11	-2.87	-3.04	2.51	0.26	2.12	-2.12	0.99	-0.44	2.01	3.40	-0.39	0.02	-0.99	-1.77	1.21	-0.28
SOLO	0.76	1.75	0.19	-0.88	1.69	-0.65	-4.31	-0.06	2.38	-0.37	3.70	-0.93	0.17	3.19	2.36	1.46
OP10	-4.35	2.03	-3.48	0.53	-2.30	0.65	0.65	1.92	-5.33	-1.74	2.05	-2.68	-3.12	-0.28	0.02	-2.10
KD9	-2.16	-1.43	1.88	-0.10	1.28	-3.04	1.63	-0.09	1.97	3.98	-0.88	-0.43	3.59	-0.99	1.60	0.55
OP11	-1.66	2.05	-0.66	2.82	-5.63	-0.16	-0.96	1.40	-1.31	-2.02	-2.84	-1.19	-0.26	0.27	1.33	-2.00

*=p<0.01

**=p<0.001

^{ns}=not significant

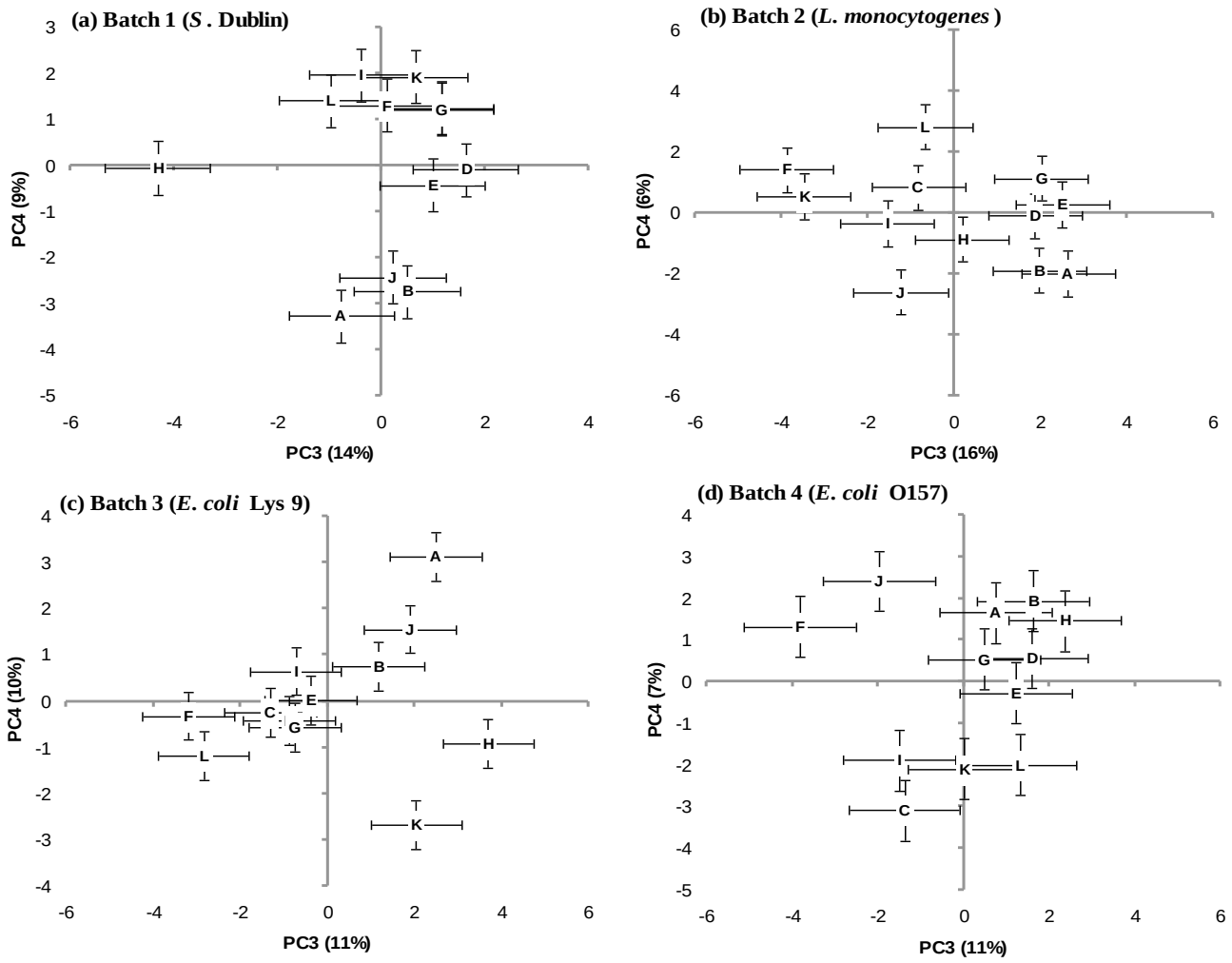


Fig. 3B Ordination of soils according to third and fourth principal components derived from individual PLFA profiles for each pathogen batch at respective T_0's for (a) *S.* Dublin, (b) *L. monocytogenes*; (c) *E. coli* Lys 9 and (d) *E. coli* O157. Data represent PC scores ± standard error (n=3). See Table 6.1 for soil identification

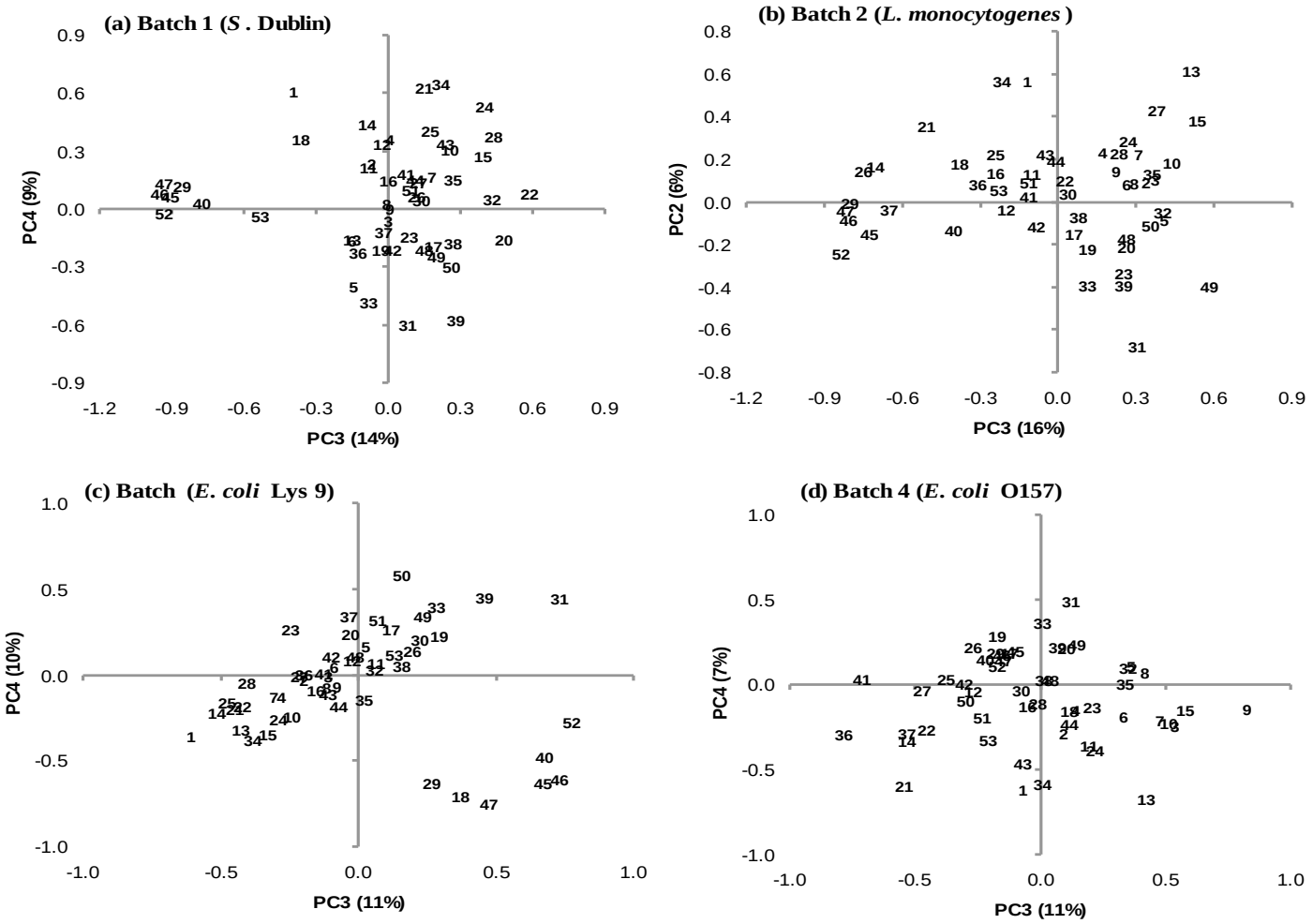


Fig. 3C Loading plots associated with third and fourth principal components derived from individual PLFA profiles for each pathogen batch at respective T_0's for (a) *S.* Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157. Data represent PC loadings (n=3). See Table 6.2 for PLFA identification

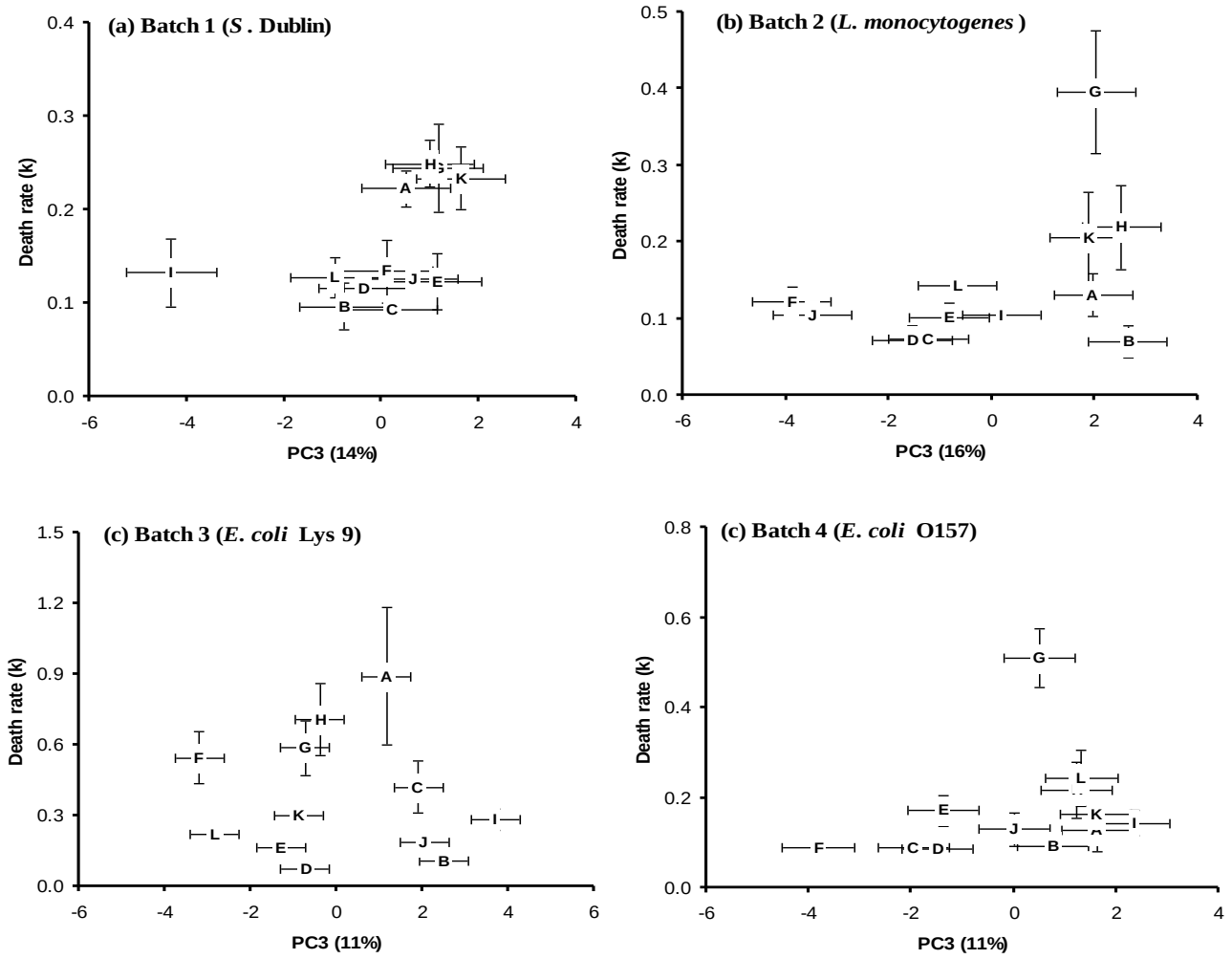


Fig. 3D Relationship between death rate of (a) *S*. Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157 and community structure represented by third principal component derived from average PLFA profiles associated with each batch. Data represent average values ± standard error (n=3). See Table 6.1 for soil identification

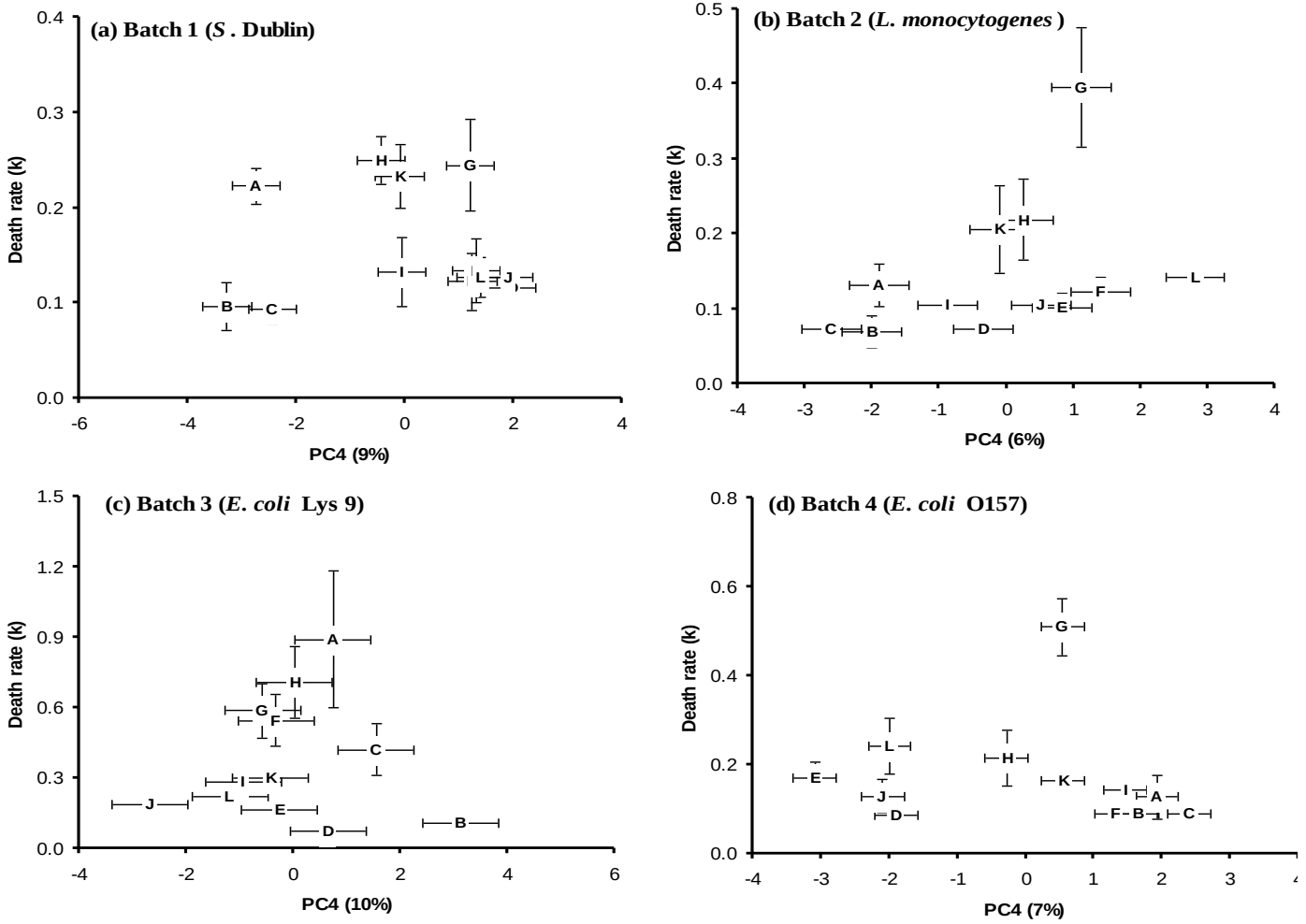


Fig. 3E Relationship between death rate of (a) *S.* Dublin, (b) *L. monocytogenes*, (c) *E. coli* Lys 9 and (d) *E. coli* O157 and community structure represented by fourth principal component derived from average PLFA profiles associated with each batch. Data represent average values ± standard error (n=3). See Table 6.1 for soil identification

Glossary of Terms

Abiotic

Physical rather than biological; not derived from living organisms

Adsorption

The accumulation of gases, liquids, or solutes on the surface of a solid or liquid

Aeration

To supply with air or expose to the circulation of air

Aerosol

A substance enclosed under pressure and able to be released as a fine spray, typically by means of a propellant gas

Amensilism

A symbiotic relationship between organisms in which one species is harmed or inhibited and the other species is unaffected

Amoeba

Any of various one-celled aquatic or parasitic protozoans of the genus Amoeba or related genera, having no definite form and consisting of a mass of protoplasm containing one or more nuclei surrounded by a flexible outer membrane

Antagonism

Inhibition of or interference with the action of a substance or organism by another

Antibiosis

The antagonistic association between an organism and the metabolic substances produced by another

Antibiotic

A substance that inhibits the growth of or destroys microorganisms

Antigen

A substance that when introduced into the body stimulates the production of an antibody

Asymptomatic

Neither causing nor exhibiting symptoms of disease

Bacteria

A member of a large group of unicellular microorganisms lacking organelles and an organized nucleus, including some that can cause disease

Bacteriodetes

A phylum of bacteria comprised of three classes: Bacteroides, Flavobacteria, and Sphingobacteria

Bacteriophage

A virus that infects and lyses certain bacteria

Biocontrol

Control of pests by disrupting their ecological status, as through the use of organisms that are natural predators, parasites, or pathogens

Bioremediation

The branch of biotechnology that uses biological process to overcome environmental problems

Biosolid

Solid or semisolid material obtained from treated wastewater, often used as fertilizer

Biotic

Of, relating to, or resulting from living things, esp. in their ecological relations

Bioturbation

The stirring or mixing of sediment or soil by organisms, especially by burrowing or boring

Buccal cavity

The cavity between the jaws and the cheeks

Carrying capacity

The maximum, equilibrium number of organisms of a particular species that can be supported indefinitely in a given environment

Catabolic function

The metabolic breakdown of complex molecules into simpler ones, often resulting in a release of energy

Cell lysis

The dissolution or destruction of cells, such as blood cells or bacteria, as by the action of a specific lysin that disrupts the cell membrane

Chromosome

A threadlike linear strand of DNA and associated proteins in the nucleus of eukaryotic cells that carries the genes and functions in the transmission of hereditary information

Ciliate

A single-celled animal of the phylum Ciliophora (kingdom Protista), distinguished by the possession of cilia or ciliary structures

Commensal

One of several types of bacteria that normally inhabit the intestine of humans and animals

Competitive exclusion

The inevitable elimination from a habitat of one of two different species with identical needs for resources

Conjugative

Of, relating to, or producing combination or conjunction; combinative or connective

Coxsackievirus

Any of a group of enteroviruses that cause various respiratory, neurological, and muscular diseases in humans

Cyst

Infectious form of many protozoan parasites usually passed in the faeces and provided with a highly condensed cytoplasm and resistant cell wall

Cytotoxin

A substance toxic to cells

Desorption

The release or removal of an adsorbed material from the surface of a solid adsorbent

Echovirus

Any of a group of enteroviruses that can cause a range of diseases, including respiratory infections and a mild form of meningitis

Edaphic

Of, produced by, or influenced by the soil

Eukaryote

A single-celled or multicellular organism whose cells contain a distinct membrane-bound nucleus

Excystation

Escape from a cyst or envelope, as in that stage in the life cycle of parasites occurring after the cystic form has been swallowed by the host

Facultative anaerobe

An organism, such as a bacterium, that can live in the absence as well as in the presence of atmospheric oxygen

Faecal coliform

A faecal coliform is a facultatively-anaerobic, rod-shaped, gram-negative, non-sporulating bacterium

Flagellate

A protozoan that has one or more flagella used for swimming

Food vacuoles

A vacuole with a digestive function in the protoplasm of a protozoan

Friability

The condition of being easily crumbled or pulverized

Fungal hyphae

Very fine filaments produced within soils by fungi through which exchanges of nutrients and organic products occur

Gastrointestinal tract

Tubular passage of mucous membrane and muscle extending from mouth to anus, functions in digestion and elimination

Genotype

The genetic constitution of an individual organism

Gram negative

Of, relating to, or being a bacterium that does not retain the violet stain used in Gram's method

Gram positive

Of, relating to, or being a bacterium that retains the violet stain used in Gram's method

Hydrophilic

Having an affinity for water, readily absorbing or dissolving in water

Hydrophobic

Repelling, tending not to combine with, or incapable of dissolving in water

Infective dose

Number of microorganisms that would initiate an immunological response by a host

Inoculum

A substance used for inoculation

K-strategist

Species of organism that uses a survival and reproductive 'strategy' characterised by low fecundity, low mortality, longer life and with populations approaching the carrying capacity of the environment, controlled by density-dependent factors

Luminescence

The emission of light by a substance that has not been heated

Lysogeny

The fusion of the nucleic acid of a bacteriophage with that of a host bacterium so that the potential exists for the newly integrated genetic material to be transmitted to daughter cells at each subsequent cell division

Lytic

Infection with a bacteriophage that ends in lysis of the host cell

Mesocosm

Any system larger than a microcosm but smaller than a macrocosm

Metabolite

A substance formed in or necessary for metabolism

Methanotroph

A bacterial organism that can use methane as its only source of carbon and energy

Microbial spore

A bacteria that, because of its thick outer wall, is easily able to survive in hostile environments otherwise not conducive to bacterial growth and reproduction

Microbiostasis

Inhibition of the growth or multiplication of microbiota

Microcosm

A small, representative system having analogies to a larger system in constitution, configuration, or development

Morphology

The form and structure of organisms

Mucous

The viscous, slippery substance that consists chiefly of mucin, water, cells, and inorganic salts and is secreted as a protective lubricant coating by cells and glands of the mucous membranes

Nematode

Any of several worms of the phylum Nematoda, having unsegmented, cylindrical bodies, often narrowing at each end

Niche space

The position of a particular species or population in an ecological community

Nucleic acid

Any of a group of complex compounds found in all living cells and viruses, composed of purines, pyrimidines, carbohydrates, and phosphoric acid

Obligate anaerobe

An organism that cannot grow in the presence of oxygen

Obligate parasite

An organism that grows, feeds, and is sheltered on or in a different organism while contributing nothing to the survival of its host

Oligotrophic

Lacking in plant nutrients and having a large amount of dissolved oxygen throughout

Oocyst

A thick-walled structure in which sporozoan zygotes develop and that serves to transfer them to new hosts

Opportunistic pathogen

Organism that exists harmlessly as part of the normal human body environment and does not become a health threat until the body's immune system fails

Pathogenicity

Capable of causing disease

Phenotype

The set of observable characteristics of an individual resulting from the interaction of its genotype with the environment

Phylogeny

The evolutionary development and history of a species or higher taxonomic grouping of organisms

Physiology

The way in which a living organism or bodily part functions

Phytopathogen

An organism that is pathogenic to a plant

Plasmid

A circular, double-stranded unit of DNA that replicates within a cell independently of the chromosomal DNA

Poliovirus

Any of a group of enteroviruses, including poliomyelitis

Predation

The capturing of prey as a means of maintaining life

Preferential flow

Leaching phenomenon whereby water and a dissolved pesticide percolating down through the soil profile move more rapidly through soil macropores or sand/gravel lens than through the network-of-smaller-pores in-the bulk soil

Prokaryote

An organism of the kingdom Prokaryotae comprising the bacteria and cyanobacteria, characterized by the absence of a distinct, membrane-bound nucleus or membrane-bound organelles, and by DNA that is not organized into chromosomes

Proteolytic enzyme

Any enzyme that catalyzes the splitting of proteins into smaller peptide fractions and amino acids

Protozoa

Any of a large group of single-celled, usually microscopic, eukaryotic organisms, such as amoebas, ciliates, flagellates, and sporozoans

R-strategist

Species of organism that uses a survival and reproductive 'strategy' characterised by high fecundity, high mortality, short longevity; populations controlled by density-independent factors

Recombinant

Of, relating to, or denoting an organism, cell, or genetic material formed by recombination

Rhizosphere

The region of soil in the vicinity of plant roots

Ribosome

A minute particle consisting of RNA and associated proteins, found in large numbers in the cytoplasm of living cells

Rotavirus

The reovirus causing infant enteritis

Rumen

The first division of the stomach of a ruminant animal, in which most food collects immediately after being swallowed and from which it is later returned to the mouth as cud for thorough chewing

Serotype

A group of closely related microorganisms distinguished by a characteristic set of antigens

Shiga-toxin

Toxins produced by certain strains of Escherichia coli which disrupt the function of the ribosome

Soil microbial biomass

The total amount of organisms in the soil, excluding macrofauna and plant roots

Soil microbial community structure

Configuration of microorganisms within the soil matrix

Soil microbial diversity

Expression of the variety of soil microorganisms and activities at the genetic, species, and soil ecosystem levels

Somatic coliphage

A bacteriophage that infects coli bacteria by attaching directly to the outer cell membrane or cell wall

Sporozoite

A motile sporelike stage in the life cycle of some parasitic sporozoans that is typically the infective agent introduced into a host

Substrate

The surface or material on or from which an organism lives, grows, or obtains its nourishment

Synergism

Interaction of discrete agents such that the total effect is greater than the sum of the individual effects

Systemic

Relating to or affecting the entire body or an entire organism

Trophozoite

A protozoan, especially of the class Sporozoa, in the active stage of its life cycle

Vector

A carrier that transfers an infective agent from one host to another

Vero cell

Vero cells are lineages of cells used in cell cultures

Virulent

Extremely infectious, malignant, or poisonous

Virus

A microorganism that is smaller than a bacterium that cannot grow or reproduce apart from a living cell

Virus capsid

The protein coat or shell of a virus particle, surrounding the nucleic acid or nucleoprotein core

Xenobiotic

Relating to or denoting a substance, typically a synthetic chemical, that is foreign to the body or to an ecological system

Zoonosis

A disease that can be transmitted to humans from animals